Effects of intra-uterine infusion of lipopolysaccharide on the inflammatory response, endometrial gene expression, uterine health, and reproductive performance of dairy cows diagnosed with vaginal purulent discharge

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Dedication

I dedicate my dissertation work to my family and friends. Particularly to my father Adelio Jose de Moraes, my mother Marcia Rogelia Nascimento Moraes, my sister Nayara Nascimento Moraes, and my girlfriend Fernanda Miyagaki Shoyama for their love and support during every step of the way. I could not be more grateful for having them in my life.

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CHAPTER I

A Literature Review

Introduction

Profitability in dairy farms is greatly affected by the reproductive performance of the herd (Britt, 1985). Although there are many factors affecting reproductive efficiency in dairy cattle, in this dissertation we will focus on postpartum uterine diseases, especially endometritis and vaginal purulent discharge.

During the transition period that corresponds to the three weeks before to three weeks after parturition, cows undergo significant changes in feed intake, energy balance (Grummer et al., 2004) and metabolic and hormonal profiles (Drackley, 1999). These changes predispose postpartum lactating dairy cows to immunodeficiency and to uterine diseases (LeBlanc, 2008).

Uterine bacterial contamination occurs in 80-90% of dairy cows during the first two weeks after parturition (Sheldon et al., 2009). On the first weeks after calving, there is a cycle of bacterial contamination, clearance and re-contamination of the uterine lumen. Most cows are able to solve the uterine bacterial contamination during this period, but approximately 10-20% of them will fail in this process and will develop clinical endometritis (Potter et al., 2010). Occurrence of uterine infections (i.e. metritis and endometritis) is a function of amount and pathogenicity of bacteria to which the uterus is exposed to and immune function (Hansen, 2013).

Sheldon et al. (2006) classified uterine diseases in the following 5 categories: puerperal metritis, metritis, clinical endometritis, subclinical endometritis and pyometra. These uterine diseases are highly prevalent in high producing dairy cows (Galvão, 2012) and have a negative economic impact on the dairy industry consequent to infertility, increased culling for failure to conceive, reduced milk production, and cost of treatment (Sheldon et al., 2009).

Galvão (2012) reported that the risk factors associated with metritis are primiparity, dystocia, twin births, retained placenta (**RP**), stillbirth, abortion, prolapsed uterus and ketosis. Similarly, risk factors associated with endometritis are dystocia, twins, RP, stillbirth, abortion, metritis, vulva conformation, male offspring, and ketosis.

Postpartum Microbiota of the Uterus

The most prevalent bacteria isolated from uterine lumen of cows with uterine infection are *Escherichia coli* and *Trueperella pyogenes* (Sheldon et al., 2009). *Escherichia coli* is a gram negative bacteria that resides in cow's digestive tract as part of the normal flora. After parturition, the presence of lochia in the uterus combined with intrauterine temperature and pH, create a favorable environment for bacterial multiplication (Hussain et al., 1990). *Escherichia coli* is one of the most prevalent bacteria isolated in the first days after calving (Hussain et al., 1990) and is reported to precede infection with *T. pyogenes*, which is isolated from cows with severe clinical endometritis (Williams et al., 2007).

Trueperella pyogenes expresses several virulence factors that result in severe endometrial lesions. Pyolysin (**PLO**), a potent extracellular toxin, is a primary virulence

factor produced by *T. pyogenes*. Pyolysin, is a haemolysin because it has the capacity of lysing red blood cells. Moreover, PLO is cytolytic to several cell types including polymorphonuclear leukocytes (**PMNL**) and macrophages. The mechanism for the PLO cytolytic effect is through formation of large pores in eukaryotic cell membranes, leading to osmotic death (Jost and Billington, 2005).

Fusobacterium necrophorum and *Prevotella species* are anaerobes that act synergistically with *T. pyogenes* increasing likelihood and severity of uterine disease (Sheldon et al., 2009). Bonnett et al. (1991) reported that the presence of *T. pyogenes* in biopsies collected at 26 days postpartum (**DIM**) was positively correlated with isolation of anaerobic bacteria (e.g. *Clostridium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus*) and presence of segmented inflammatory cells on the same biopsy. Moreover, authors demonstrated a strong association between isolation of *T. pyogenes* and anaerobic bacteria 26 DIM and increased histological abnormalities in uterine biopsies collected 40 DIM.

Williams et al. (2005) categorized bacterial isolates form 328 swabs collected from uterine lumen of dairy cows 21 and 28 DIM in three categories according to their expected pathogenic potential. The first category comprises recognized uterine pathogens associated with uterine endometrial lesions and included: *T. pyogenes*, *P. melaninogenicus*, *E. coli* and *F. necrophorum*. The second category is formed by potential pathogens frequently isolated from the bovine uterine lumen and cases of endometritis not commonly associated with uterine lesions: *Bacillus licheniformis*, *Enterococcus faecalis*, *M. haemolytica*, *Pasteurella multocida*, *Peptostreptococcus*

species, *Staphylococcus aureus* and non-haemolytic Streptococci. The third category is formed by opportunistic contaminants transiently isolated from the uterine lumen and not associated with endometritis: *Clostridium perfringens*, *Klebsiella pneumonia*, *Micrococcus species*, *Providencia stuartii*, *Proteus species*, *Staphylococcus species* (coagulase negative), *α-haemoltyic Streptococci*, *Streptococcus acidominimus*, and *Aspergillus species*. Bovine herpervirus 4 is the only virus consistently associated with uterine diseases in postpartum cows and its presence is normally concurrent with bacteria that are known to cause uterine disease (Sheldon et al., 2009).

Endometritis Diagnosis

Clinical endometritis is characterized in cattle as the presence of a purulent (> 50% pus) uterine discharge detectable in the vagina after 21 DIM or a mucopurulent discharge (approximately 50% pus and 50% mucus) detectable in the vagina after 26 DIM without any systemic symptoms (Sheldon et al., 2006).

The diagnosis of clinical endometritis can be made simply by visualization of fresh and characteristic discharge from the vulva or on the perineum and tail (LeBlanc et al., 2002). If discharge is not visible externally, which is often the case, the diagnosis can be made through an evaluation of the vaginal discharge using vaginoscopy (LeBlanc et al., 2002), the gloved hand (Sheldon et al., 2002), or the Metricheck device (McDougall et al., 2007). When performing any of these three methods, the vulva needs to be well cleansed in order to avoid introduction of contaminants into the vagina (Galvão, 2012) and the instruments used must be clean and lubricated.

For the vaginoscopic examination, the vaginoscope is inserted into the vagina until it touches the external os of the cervix (the opening of the lower part of the cervix that protrudes into the vagina). Cervix and vagina are then visually examined with illumination from a penlight for presence and quality of the discharge (LeBlanc et al., 2002).

In the gloved hand method, vaginal discharge is scooped by hand. A gloved hand is inserted through the vulva, and the lateral, dorsal and ventral walls of the vagina and the external cervical os are palpated. Vaginal discharge is then withdrawn for examination (Sheldon et al., 2002).

A fairly new device called Metricheck has also been used to diagnose endometritis in cattle. This device was invented in New Zealand (Simcro, New Zealand) but has become popular around the world because it is easier and faster than vaginoscopy. The metricheck device consists of a stainless steel rod with a semi-spherical rubber cup that is used to retrieve vaginal contents. To examine a cow, the device is introduced through the vulva into the cranial vagina to scoop any vaginal discharge from the anterior vagina (Runciman et al., 2009).

The efficacy of vaginoscopy and metricheck to diagnose clinical endometritis were compared (McDougall et al., 2007; Runciman et al., 2009). McDougall et al. (2007) examined 191 cows with more than 14 DIM and history of periparturient disease for clinical endometritis using both the metricheck and the vaginoscope. The order of examination (metricheck first vs. vaginoscope first) was randomized within each herd. Three analytical approaches were used to estimate test sensitivity and specificity for

detection of endometritis in absence of a gold standard. In all three approaches, the metricheck had a higher sensitivity but lower specificity than vaginoscopy. Using the maximum likelihood approach, for instance, sensitivity was 96.2% vs. 71.5% and specificity was 77.6% vs. 87.1% for metricheck and vaginoscopy, respectively.

Runciman et al. (2009) examined 423 postpartum dairy cows with both metricheck and vaginoscopy between 7 and 28 DIM. Vaginally retrieved exudate was scored from 0 (clear or absent) to 3 (purulent). Vaginal exudate score from individual cows obtained with Metricheck and vaginoscopy were compared using a quadratic weighted kappa and agreement between the two methods was substantial [kappa = 0.73 (95% CI 0.66–0.80)] when assessing the status (pus or no pus) of the discharge. Authors suggested that the diagnosis of purulent exudate in the vagina of dairy cows using the Metricheck might be better than using vaginoscope because the Metricheck is quicker and easier than vaginoscopy and there is no difference in sensitivity for detection of purulent or mucopurulent vaginal discharge between the two techniques.

Pleticha et al. (2009) evaluated the incidence of endometritis in 2 German dairy herds based on the metricheck, gloved hand, and vaginoscopy. Cows (n = 1,002) were examined for clinical endometritis 21 to 27 DIM using one of the three methods and those diagnosed with endometritis were treated with an analogue of prostaglandin (**PG**) $F_{2\alpha}$ twice, 14 d apart. Incidence of clinical endometritis was significantly greater in cows examined with Metricheck (47.5%) compared with cows examined with vaginoscopy (36.9%) and gloved hand (36.8%). There were no differences in reproductive performances of cows according to diagnostic method. Authors suggested that the lack of

differences among diagnostic methods in regards to reproductive performance may have been a consequence of low specificity of the Metricheck method or inefficiency of $PGF_{2\alpha}$ in resolving endometritis.

The definitive diagnosis of endometritis is made through histological examination of endometrial biopsies (Sheldon et al., 2006). According to Bondurant (1999), endometritis is histologically characterized by disruption of surface epithelium, inflammatory cells infiltration, vascular congestion, stromal edema, and by varying degrees of lymphocyte T and B cells accumulation in the superficial layers. The downsides of this procedure is that it is costly, time consuming, may depress fertility (Sheldon et al., 2006), and well trained personnel are required. Bonnett et al. (1991) described the procedure for endometrial biopsies and histological assessment. Briefly, biopsy of endometrium may be performed using a 3/8 inch diameter biopsy punch (Equi Bov, Campbellville, Ontario). Biopsies are taken from the dorsomedial region of the horn, just anterior to the bifurcation, from the larger horn that is presumed to be the previously gravid horn. Biopsies are then gently removed from the biopsy instrument and transferred to a vial of Bouin's solution. After two to four hours, the samples are transferred to 70% alcohol. After that, sections are cut and processed routinely and stained with hematoxylin and eosin.

Ultrasonography and rectal palpation are also useful tools for examining cows for endometritis. In a study conducted by LeBlanc et al. (2002), cows with cervical diameter > 7.5 cm after 20 DIM had impaired reproductive performance and were considered to have had clinical endometritis. Mateus et al. (2002) evaluated the use of ultrasonography

to measure uterine body diameter, intrauterine fluid volume (scored from 0 = no fluid; 1-3 = increasing spots of fluid) and ovarian function of 26 dairy cows from parturition to the sixth week postpartum. Cows were allocated to one of three categories according to uterine lochia: healthy group (normal lochia, n = 7), mild endometritis (purulent lochia, n = 11), and severe endometritis (heavy, fetid and purulent lochia with or without systemic symptoms, n = 8). Cows with severe endometritis had significantly larger uterine body diameter than the other categories 3 and 4 weeks postpartum. Cows with severe endometritis had significantly larger intrauterine fluid volume than healthy and mildly endometritic cows 2, 5 and 6 weeks postpartum. In addition, cows with mild endometritis had numerically higher score for intrauterine fluid volume from 0 to 5 weeks postpartum, but the difference was significant only in week 3. Cows with severe endometritis were more likely to have prolonged anoestrus, prolonged luteal phases and ovarian cysts than healthy cows.

Subclinical endometritis

Subclinical endometritis is an inflammation of the endometrium without presence of purulent discharge in the vagina. The diagnoses of subclinical endometritis can be done by measuring the proportion of neutrophils present in uterine cytology samples collected using the cytobrush (Kasimanickam et al., 2004) or the low-volume uterine lavage procedure (Gilbert et al., 2005).

In the uterine cytology technique, a cytobrush (VWR Canlab, Mississauga, ON, Canada) designed for humans is used (Kasimanickam et al., 2004). In order to use a human cytobrush in cows, the plastic handle of the cytobrush is cut to 3 cm in length,

threaded onto a solid stainless steel rod (65 cm in length and 4 mm in diameter), and placed in a stainless steel tube (50 cm in length and 5 mm in diameter). The stainless steel instrument is protected with a sanitary plastic sleeve to avoid contamination of the cytobrush with vaginal content. After cleaning the vulva, the lubricated instrument is inserted into the vagina and directed to the external cervical os, the sanitary sleeve is punctured, and the instrument advanced through the cervix into the base of the larger horn, presumably the previously gravid horn. The cytobrush is then exposed and uterine sample is collected by rotating the cytobrush in a clockwise direction while in contact with the uterine wall. The cytobrush is then retracted into the stainless steel tube, the instrument is removed, and the slide is prepared by rotating the cytobrush in a glass microscope slide. Subclinical endometritis can also be diagnosed using the low-volume uterine lavage procedure. In this technique, 20 mL of sterile saline solution is infused into the uterus using a sterile infusion pipette protected by a sanitary sleeve. The infusion pipette is introduced through the vulva and directed to the cervix, and the sanitary sleeve is punctured while the infusion pipette is inside the cervix, and then the pipette is placed into the uterine body. Upon infusion, the uterus needs to be massaged, and at least 5 mL of the infused fluid is aspirated. Aspirated fluid must be processed within 6 hours. Samples need to be centrifuged using either a conventional or cytospin centrifuge. When using a conventional centrifuge, most of the supernatant is discarded and just one remaining drop is used to prepare the smear on a microscope glass slide. If using a cytospin centrifuge, approximately 150 µl of aspirated fluid is transferred to a cytospin container and samples are centrifuged onto glass slides at 700 *g* for 5 min. Slides are air-

dried, stained using Diff-Quik, and read under 400x magnification. Endometrial inflammation is determined based on the percentage of PMNL in the smear after counting a minimum of 100 cells, excluding erythrocytes (Gilbert et al., 2005; Galvão, 2012).

Kasimanickam et al. (2004) evaluated the cytobrush technique and ultrasonography to diagnose subclinical endometritis in postpartum dairy cows. Holstein cows were examined for vaginal discharge using a vaginoscope between 20 and 33 DIM and 228 clinically normal cows were enrolled in the experiment. These clinically normal cows were further examined by ultrasonography and endometrial cytology 20-33 DIM and 14 days later and their reproductive performance was evaluated for a minimum of 8 months or until diagnosed pregnant or culled. Cows with > 18% PMNL in uterine cytology 20-33 DIM [AHR (95% CI) = 0.59 (0.43, 0.81)] and cows with > 10% PMNL in uterine cytology 34-47 DIM [AHR (95% CI) = 0.56 (0.41, 0.78)] had reduced adjusted pregnancy hazard ratio than cows with < 18% PMNL in uterine cytology 20-33 DIM and cows with < 10% PMNL in uterine cytology 34-47 DIM. In regards to the ultrasonography examination, cows that had intrauterine fluid at 20-33 DIM [AHR (95% CI) = 0.46 (0.33, 0.74)] and at 34-47 DIM [AHR (95% CI) = 0.58 (0.36, 0.94)] had reduced adjusted pregnancy hazard ratio than cows with no intrauterine fluid in each examination. Therefore, presence of fluid or > 18% PMNL in uterine cytology 20-33 DIM and presence of fluid or > 10% PMNL in uterine cytology 34-47 DIM are associated with compromised reproductive performance.

In a subsequent experiment the cytobrush and the uterine lavage techniques were compared for diagnosis of sub-clinical endometritis (Kasimanickam et al., 2005).

Holstein cows (n = 35) that did not have abnormal vaginal discharge based on vaginoscopy 20 to 33 DIM were used in the study. Endometrial samples were collected from all cows by the cytobrush technique followed by the lavage technique between 20 and 33 DIM and between 34 and 47 DIM. The cytobrush technique resulted in significantly higher percentage of PMNL in uterine cytology than the lavage technique between 20 and 33 DIM but no differences were observed between the cytobrush and lavage techniques between 34 and 47 DIM. There was a negative correlation between uterine diameter and fluid recovery by the lavage technique. Therefore, authors speculated that the observed differences between cytobrush and lavage techniques between 20 and 33 DIM were because of the larger uterine volume and reduced cell recovery. Authors concluded that cytobrush technique is a more consistent and reliable method than the uterine lavage to obtain endometrial cytology samples from postpartum dairy cows.

Ghasemi et al. (2012) suggested that endometrial inflammation can be predicted by measuring cytokine gene expression in endometrial cytobrush samples. In this experiment, cows were examined for subclinical endometritis at 28-41 DIM using the uterine cytology technique, and cows with > 18% of PMNL in uterine cytology were considered to have sub-clinical endometritis. Cytobrush from cows with subclinical endometritis contained a 20-fold higher tumor necrosis factor alpha (**TNFα**), a 30-fold higher interleukin (**IL**) 6, and a greater than 50-fold higher IL-8 mRNA expression than cytobrush from healthy cows. Even though the authors suggested that IL-8 gene expression in cytobrush might be a useful tool to predict endometrial inflammation in

cattle, sample size in this experiment was not sufficient to calculate sensitivity and specificity.

Endometritis Treatment

There are no commercial products currently approved for treatment of endometritis in dairy cows in the United States. However, intrauterine treatment of endometritic cows using 500 mg of cephapirin benzathine in 19 g ointment base (Metricure®, Intervet, Boxmeer, The Netherlands) is approved in Canada, Europe and New Zealand (Galvão, 2012). LeBlanc et al. (2002) compared the reproductive performances of endometritic cows treated, at 20 and 33 DIM, with Metricure®, or intramuscular (i.m.) injection of 500 μg of cloprostenol, or not treated. Intrauterine treatment of endometritic cows with Metricure® improved pregnancy rates by 63% compared with untreated cows but only when cows were treated between 27 and 33 DIM. There were no differences in reproductive performance between $PGF_{2\alpha}$ treated endometritic cows and untreated endometritic cows. Kasimanickam et al. (2005) demonstrated improvements in reproductive performance of cows with subclinical endometritis treated with Metricure® and cloprostenol at 20 to 33 DIM. On the other hand, Dubuc et al. (2011) demonstrated that treatment of cows with clinical and subclinical endometritis with $PGF_{2\alpha}$ at both 35 and 49 DIM did not improve reproductive performance. Similarly, Lima et al. (2013) observed no benefit of a single $PGF_{2\alpha}$ treatment at 36 to 42 DIM, or 2 treatments with $PGF_{2\alpha}$ at 22-28 DIM and 36-42 DIM in uterine health or reproductive performance. Treatment of endometritic cows with $PGF_{2\alpha}$ has been suggested to improve uterine health by inducing estrus in cows with a corpus

luteum, resulting in physical expulsion of bacteria, increased concentrations of pro-inflammatory substances in the uterus (i.e. $PGF_{2\alpha}$ and estradiol), and reduced progesterone concentrations eliminating the immunosuppressive effects of progesterone in the uterus (Galvão, 2012). Progesterone is believed to cause immunosuppression in part due to the down-regulation of expression of molecules associated with bacterial recognition (i.e. tool-like receptor (**TLR**) 4), reduced expression of mRNA of pro-inflammatory genes (CXCL5, IL-1β, and IL-8; Fischer et al., 2010), and inhibition of endometrial stroma and epithelial cells' production of PGE and PGF in response to lipopolysaccharides (**LPS**; Herath et al., 2006). Up to date, however, the only treatment that appears to effectively improve fertility of endometritic cows is the intra-uterine treatment with Metricure®.

Effects of Uterine Diseases on Ovarian Function

The presence of postpartum uterine infection is associated with impaired ovarian function (Sheldon et al., 2002; Sheldon et al., 2009; Williams et al., 2007; Williams et al., 2008). The negative effect of uterine microbes on ovarian function is a complex mechanism involving the innate immune system and inflammatory mediators that are believed to interact with the hypothalamus, pituitary and ovary (Sheldon et al., 2009).

Beginning during parturition, cows have the uterus contaminated by a variety of bacteria, both gram-positive and gram-negative aerobes and anaerobes (Azawi, 2008). The outer membrane of cell walls of gram-negative bacteria is formed by proteins, phospholipids, and by its major component, LPS (Beveridge, 1999). Lipopolysaccharides suppress hypothalamic release of gonadotropin-releasing hormone (**GnRH**), reduce the

sensitivity of the pituitary to GnRH, and reduce the secretion of luteinizing hormone by the pituitary (Sheldon et al., 2009). Interestingly, follicle-stimulating hormone concentration is not affected by uterine bacterial contamination, and therefore the emergence of ovarian follicular waves is not compromised in animals with uterine disease (Sheldon et al., 2002).

Lipopolysaccharides also have a direct negative effect in the ovarian function. Williams et al. (2008), using cells collected from bovine ovaries, reported reduced granulosa cell estradiol secretion when cells were treated with LPS. Also, treatment of theca and granulosa cells with TNFα, an important pro-inflammatory cytokine produced after LPS stimulation, resulted in decreased theca cell production of androstenedione and granulosa cell production of estradiol. Intrauterine infusion of Holstein heifers with 3 μg/kg of LPS or 0.1 μg of rhTNFα resulted in fewer ovulations than heifers treated with sterile phosphate-buffered saline and, among heifers that ovulated, progesterone concentration was lower in heifers treated with LPS. Furthermore, cows with elevated uterine bacterial contamination 7 DIM had smaller dominant follicles and reduced estradiol concentration during the first postpartum follicular wave and smaller corpora lutea and progesterone concentration after ovulation (Williams et al., 2007). Luteal phase may also be disrupted and shortened in cows with endometritis. Endometrial cells challenged with LPS had greater secretion of PG, with a greater PGE to PGF ratio (Herath et al., 2006; Sheldon et al., 2009). During the normal estrus cycle luteolysis is caused by secretion in pulses of $PGF_{2\alpha}$ by the endometrium at approximately day 17 of the estrus cycle in non-pregnant cows (Miyamoto and Shirasuna, 2009). On the other

hand, PGE is luteotropic (Manns et al., 1985; Davidson et al., 1996) and intrauterine infusion of heifers with PGE_2 resulted in extended luteal phase (Thibodeaux et al., 1992). Therefore, endometritis may predispose cows to pyometra because the increased PGE to PGF ratio associated with endometritis would prolong the maintenance of the corpus luteum, an ideal condition for maintenance and worsening of uterine bacterial infections.

Lipopolysaccharide Recognition by the Innate Immune System

Innate immunity is the first line of host defense against infections, and plays an important role in pathogen recognition and triggering the subsequent inflammatory response. Cells from the innate immune system are capable of recognizing host self and nonself molecules through receptors called pattern recognition receptors (**PRR**; Mogensen, 2009). These receptors recognize molecular structures that are conserved through several classes of microbes, called pathogen-associated molecular patterns (**PAMP**). Pathogen-associated molecular patterns are essential for the survival of the microbe and, therefore, are not subject to variability, as changes in these structures are lethal to the microbe (Medzhitov and Janeway, 1997).

One of the most studied PAMP is the LPS, constituent of the outer membrane of gram-negative bacteria cell wall. The LPS molecule is composed of three structural sections: an amphipathic Lipid A, a hydrophilic polysaccharide of the core, and O-specific chain (Figure 1). The Lipid A component is responsible for the endotoxic principle of LPS. Chemically, Lipid A consists of phosphorylated glucosamine disaccharide units with long-chain fatty-acids attached. The core polysaccharide is formed by two characteristic sugars [ketodeoxyoctanoic acid (**KDO**) and a heptose] and

connects the Lipid A and O-specific chain sections. The core region can be subdivided into an outer and an inner portion, where the inner core is well conserved among all gram-negative bacteria, being essential for LPS structure and bacterial viability. The third section, O-specific chain, is formed by a polymer of oligosaccharides with repeating units of glycosyl residues (Rietschel et al., 1994). The O-specific chain is the most chemically diverse component of the LPS outer membrane, varying among even the same species of bacteria (Kalynych et al., 2012) and, therefore, functioning as an important surface antigen, also referred to as the O-antigen.

In mammals, the physiological recognition of bacterial LPS occurs through the TLR-4 in association with myeloid differentiation factor 2 (**MD-2**; Park et al., 2009). Members of TLR family are composed by three different domains (Figure 2): an extracellular domain composed of many leucine-rich repeat (**LRR**), a membrane-spanning domain, and an intracellular domain, termed toll-interleukin-1 receptor (**TIR**) domain (Medzhitov, 2001).

Lipopolysaccharide while within bacterial membranes cannot effectively induce an immune response. To actively stimulate the immune system, LPS needs to be extracted from the bacterial membrane and be presented to the receptor complex composed of TLR4 and MD-2. For that, a serum lipid transfer protein called LPS-binding protein extracts monomers of LPS from the bacterial membrane and presents them to the innate immune receptor cluster of differentiation (**CD**) 14, expressed on myelomonocytic cells or soluble in the circulation. The role of the CD14 is to load the LPS to the receptor complex formed by TLR4 and MD-2 (Miyake, 2006).

Tool-like receptor 4 TIR domain is critical for signal transduction after LPS binding to the complex TLR4-MD-2. Toll-interleukin-1 receptor domains are composed of three highly conserved regions that play an important role in the protein-protein interactions among TLR4 and five adaptor proteins, leading to the activation of transcription factors such as nuclear factor-κB (**NF-κB**) and members of the interferon (**IFN**)-regulatory factor family (Lu et al., 2008; O'Neill and Bowie, 2007). The activation of these two factors regulates the transcription of a variety of genes, including cytokines and chemokines, being crucial for controlling the innate and adaptive immunity (Li and Verma, 2002; Schroder et al., 2004).

Lipopolysaccharides stimulate the production of pro-inflammatory cytokines, such as TNFα, IL-1, IL-6 and IL-8 (Wright, 1999), which are produced by many different cell types. Interleukin-1 and TNFα are functionally related pro-inflammatory cytokines that mediate many of the consequences of sepsis, including fever, induction of acute-phase proteins, migration of inflammatory cells, vasodilatation and stimulation of other cytokines (Marsh et al., 1994). Interleukin-6 is a cytokine with both pro and anti-inflammatory properties, and might play an important role during the transition of innate to acquired immunity. Interleukin-6, in association with IL-1β or TNFα, stimulates the production of various chemokines leading to the attraction of neutrophils in the initial phase of inflammation. However, 24-48 h after initial infiltration, neutrophils are replaced by monocytes and T cells in order to prevent tissue damage from accumulation of proteases and reactive oxygen species produced by neutrophils. Within this context, IL-6 induces neutrophil apoptosis contributing to resolution of the acute neutrophil

infiltration (Scheller et al., 2011). Interleukin-6 also upregulates expression of adhesion molecules on endothelial cells and leukocytes, favoring the process of leukocyte transmigration from blood circulation to inflamed tissues (Scheller et al., 2011). In addition to regulating the immune response, IL-6 also plays an important role in acute phase reaction and hematopoiesis (Tilg et al., 1995). Interleukin-6 acts as a hepatocyte-stimulating factor inducing production of various acute-phase proteins in the liver. Moreover, IL-6 also acts on hematopoietic stem cells and, together with IL-3, promotes the transition from a resting phase to a proliferative phase of the cell cycle. In addition, IL-6 induces maturation of megakaryocytes, resulting in an increase in platelets (Kishimot et al., 1992). Interleukin-8 is a cytokine produced by variety of cell types and is a member of the chemokine family, acting to guide leukocytes, primarily neutrophils, to the site of inflammation. Interleukin-8 also stimulates neutrophil function by increasing the ability of neutrophils to adhere to stimulated endothelial cells, increases neutrophil expression of integrin adhesion molecules LFA-1 (CD11aCD18) and MAC1 (CD11bCD18), and induces neutrophils to release lysosomal enzymes (Harada et al., 1994).

Leukocytes Migration

Leukocyte activation and migration to the site of inflammation is a crucial component of both innate and adaptive immunity. In order to transmigrate from the blood vessel into inflamed tissues, leukocytes need to interact with the endothelium (Albelda et al., 1994). Four families of cell adhesion molecules (**CAM**) play a critical role in

leukocytes endothelial interactions: the selectin family, the mucin-like family, the integrin family, and the immunoglobulin superfamily (Kindt et al., 2006).

The selectin family is responsible for the initial stickiness of leukocytes to endothelium, allowing leukocytes to roll along vascular endothelium (Kindt et al., 2006). This family includes three molecules designated by the prefixes L (leukocyte), P (platelet), and E (endothelial; Carlos and Harlan, 1994). L-selectin is expressed by hematopoietic cells, with most classes of leukocytes expressing L-selectin at some stage of differentiation (Tedder et al., 1995). P-selectin is expressed in the α-granules of platelets and in the Weibel-Palade bodies of endothelial cells (André, 2004). E-selectin, similarly to P-selectin, is expressed by endothelial cells. Thus, P-selectin and E-selectin are upregulated on endothelium surface upon an inflammatory stimulus (Binder and Ernst, 2011).

Mucins are a group of proteins with extended structure that serve as binding site for selectins. L-selectin expressed by leukocytes, for example, binds to the mucin-like molecules CD34 and GlyCAM-1 of endothelial cells. Neutrophils express a mucin-like molecule (PSGL-1) that binds to E-selectin and P-selectin expressed on endothelium (Kindt et al., 2006).

Members of the integrin family while interacting with members of immunoglobulin superfamily, play an important role in leukocytes firm adhesion and diapedeses during leukocyte emigration from the bloodstream to extravascular tissues (Carlos and Harlan, 1994). The integrin family is composed by heterodimer proteins consisting of noncovalently associated α and β subunits (Albelda and Buck, 1990). One

of the most important functions of integrins is to bind to extracellular matrix molecules and provide cell matrix interactions throughout the body (Aplin et al., 1998). Leukocytes express a subfamily of integrins called β2-integrins (CD18) that can be associated with three distinct α subunits (CD11a, CD11b, and CD11c; Mazzone and Ricevuti, 1995). The combination of integrins expressed by a cell type will permit these cells to bind to different CAMs expressed on vascular endothelium (Kindt et al., 2006).

The fourth family of CAMs is called immunoglobulin superfamily and is composed of more than 765 members, being one of the largest and most diverse proteins in the body. In this group are included the major histocompatibility complex classes I and II, cell surface glycoproteins, virus receptor and proteins of the T-cell receptor complex (Wai Wong et al., 2012). Cell surface glycoproteins include ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), and VCAM (CD106), which are expressed on vascular endothelial cells and bind to several members of the integrins family (Kindt et al., 2006).

Polymorphonuclear Leukocytes Phagocytosis and Oxidative Burst

Polymorphonuclear leukocytes, especially neutrophils, are the predominant migratory phagocytic cells present in blood and are the first cells to arrive at sites of infection. Therefore, PMNL are crucial for host defense against bacterial infections (Chang et al., 1999) and non-specific phagocytosis by neutrophils is one of the most important lines of defense against establishment of uterine infections (LeBlanc, 2008).

By definition, phagocytosis is the process of engulfing particles such as pathogens into a plasma membrane-derived vacuole, termed phagosome, which proceeds to acquire

degradative properties through action of cytotoxic agents in a process called maturation (Lee et al., 2003).

Phagocytic processes are driven by controlled rearrangement of the actin cytoskeleton (May and Machesky, 2001), which allows neutrophils to internalize both opsonized and non-opsonized particles. Opsonization enhances phagocytosis and the two principal opsonin receptors of neutrophils are Fc receptors and the β2 integrin MAC1 (Lee et al., 2003).

Fc receptors bind to Fc portion of immunoglobulin and, for PMNL, the expression of Fc receptors depends on the stage of cellular activation. In humans, the main Fc receptors are FcγRI, FcγRIIA, and FcγRIIIb. Resting PMNL express FcγR IIA and IIIb, whereas FcγRI receptors are expressed by neutrophil undergoing development and upon stimulation of neutrophils by IFN-γ and granulocyte colony-stimulating factor (McKenzie and Schreiber, 1998). Neutrophil β2 integrin MAC1 (CD11bCD18) recognizes complement-coated particles by recognizing the complement fragment C3bi (Lee et al., 2003).

Upon phagosome formation, the phagosome moves toward the interior of the cell and fuses with a lysosome, forming a phagolysosome, in which the killing and digestion of the bacteria takes place. Lysosomes are cellular organelles that contain a variety of hydrolytic enzymes that digest the ingested material (Kindt et al., 2006).

The production of reactive oxygen species, during the process called oxidative burst, plays a critical role in bacteria killing. Large amounts of superoxide are generated by a NADPH oxidase, a multisubunit entity with membrane-bound and soluble

components that assemble into a heteromeric complex when the cells are stimulated (Lee et al., 2003). Activation of NADPH oxidase can be induced by LPS, by lipoproteins, or by cytokines such as IFN-γ, IL-1β, and IL-8 (Droge, 2002). Neutrophils express several TLR on their surface, including TLR2 that detects the peptidoglycan of gram-negative bacteria, and TLR4 that recognizes LPS (Kindt et al., 2006). NADPH oxidase activation can be in response to signaling events involving TLR and FcγR (Huang et al., 2009).

Myeloperoxidase (**MPO**) is an enzyme, cationic protein, stored in large amounts in azurophilic granules of PMNL. Within the azurophilic granules, which have low pH and lack hydrogen peroxide, MPO bound to proteoglycans, mainly chondroitin-4-sulphate, are inactive. Upon bacteria phagocytosis, however, azurophilic granules discharge their content into the forming phagosome. With NADPH oxidase activation and ion influx into the phagosome, local conditions are changed, favoring the activation of MPO and other compounds present in the azurophilic granules (Arnhold and Flemmig, 2010). The combined activities of MPO and NADPH oxidase lead to the production of hypochlorous acid (**HCLO**), a potent antibacterial substance (Droge, 2002). Myeloperoxidase also regulates inflammation by promoting recruitment of PMNL and apoptosis of PMNL and other cells (Arnhold and Flemmig, 2010).

Endometrium and Innate Immunity

Endometrium is the physical barrier against establishment of uterine diseases. Endometrium possesses TLR (1 to 10) for pathogen recognition and produces antimicrobial peptides and Mucin-1 (Davies et al., 2008).

Antimicrobial peptides in mammals are divided in two main genetic categories, the cathelicidins and the defensins. Defensins in vertebrates are subdivided in three subfamilies (α, β, and θ; Selsted and Ouellette, 2005), of which β-defensins is the main sub-family and is of particular importance for mucosal immunity (Davies et al., 2008). Microbicidal effects of defensins have been demonstrated by *in vitro* studies, in which purified defensins preparations killed a wide range of microbes, including bacteria, fungi, protozoa and enveloped virus (Selsted and Ouellette, 2005).

Mucin 1 is an epithelial cell glycosylated transmembrane protein expressed in bovine endometrium that might play an important role in microbial defense by limiting microbial access to the cell surface, regulating inflammation. Interestingly, treatment of endometrial cells with LPS resulted in upregulation of expression of MUC-1 (Davies et al., 2008).

The inflammatory process that results from the contact between the endometrium and pathogens is critical for elimination of the pathogen or infection. It is well documented that cows with uterine diseases have increased endometrium gene expression of pro-inflammatory cytokines. Fischer et al. (2010) found that cows with subclinical or clinical endometritis had increased expression of CXCL5, IL-1β, IL-8 and TNFα at 21-27 DIM compared with healthy animals.

Galvão et al. (2011) evaluated endometrium mRNA gene expression of pro-inflammatory (TNFα, IL-1β, IL-6 and IL-8) and anti-inflammatory (IL-10) cytokines in cows that developed endometritis and in healthy cows at weeks 1, 3, 5 and 7 after calving. Expression of mRNA gene for TNFα and IL-1β were reduced in week 1 after

calving in cows diagnosed with endometritis 5 weeks after calving compared with healthy cows. Expression of mRNA of genes for pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) was increased in endometritic cows on weeks 5 and/or 7 after calving compared with healthy cows. There was no difference in expression of mRNA gene for IL-10 between health and endometritic cows. Tumor necrosis factor alpha and IL-1β are known to stimulate IL-8 production and expression of adhesion molecules on vascular endothelium. Therefore, TNFα and IL-1β are critical for leukocytes chemoattraction, migration and activation. The authors (Galvão et al., 2011) suggested that the decrease in expression of TNFα and IL-1β 1 week after calving in cows diagnosed with endometritis 5 weeks after calving compared with healthy cows may have compromised bacterial clearance after calving and contributed to occurrence of endometritis.

The endometrium also mediates inflammation through the production of prostaglandins. For example, LPS challenge of endometrial epithelial and stromal cells results in increased PG secretion, with an increased ratio of PGE to PGF (Herath et al., 2006). Prostaglandins of the E series modulate inflammation and immune responses and are important during homeostasis and disease processes. Prostaglandin E2 exhibits both pro- and anti-inflammatory effects. At the same time that PGE2 may induce cardinal signs of inflammation (i.e. pain, edema and fever), PGE2 may also exert strong anti-inflammatory effects by suppressing production of pro-inflammatory cytokines and enhancing the synthesis of anti-inflammatory cytokines, such as IL-10 (Cheon et al., 2006). This diversity in PGE_2 actions is believed to be the result of four PGE receptor subtypes (EP1 to EP4) coupled to different signal transduction pathways (Minami et al.,

2001). Most major cells involved in inflammation have these four subtypes of EP receptors, including lymphocytes (T and B), macrophages and dendritic cells. Depending on receptor subtype, cell population, and context of activation, PGE_2 will exert pro- or anti-inflammatory effects. For instance, PGE_2 interaction with EP4 enhances the migration of antigen-stimulated Langerhans cells to lymph nodes and subsequent T cell activation during hypersensitivity response. On the other hand, PGE_2 inhibits the production of TNFα in macrophages through the EP4 receptor and T cell proliferation through the EP2 receptor (Park and Christman, 2006).

Prostaglandins are synthetized from arachidonic acid, a fatty acid freed from phospholipids of cell walls trough action of the enzyme phospholipase A2 (PLA2), which, in the bovine endometrium, include PLA group IV (PLA2G4A and PLA2G4C) and group VI (PLA2G6; Herath et al., 2009). Tithof et al. (2007) using Western blot demonstrated that bovine endometrial epithelial cell production of $PGF_{2\alpha}$ after oxytocin stimulation is mediated trough up-regulation of PLA2G6 protein expression and activity, whereas PGE2 biosynthesis was associated with PLA2G4C protein expression and activity.

Cyclooxygenase enzymes, also known as prostaglandin-endoperoxide synthase (**PTGS**), convert arachidonic acid into PGH_2, which is quickly converted into thromboxane, prostacyclin or prostaglandins D, E or F via the action of specific prostaglandin synthase (MacKenzie et al., 2013). Interestingly, in a study conducted by Herath et al. (2009), the switch in endometrial secretion of $PGF_{2\alpha}$ to PGE after LPS challenge was associated with increased protein level of PLA2G6 rather than changes in

mRNA expression of specific prostaglandin synthase E and F. In this same study, levels of PLA2G4C protein were not affected by LPS. This effect of increased PLA2G6 after LPS challenge and accumulation of PGE is in contrast to what was reported by Tithof et al. (2007), in which the stimulation of PLA2G6 with oxytocin led to accumulation of PGF. Herath et al. (2009) speculated that the differences between studies may be a consequence of cell culture methods, the stage of ovarian cycle when the endometrial cells were collected, or differences between oxytocin and LPS challenge. Moreover, Herath et al. (2009) pointed out that in the experiment by Tithof et al. (2007), overexpression of PLA2G6 in epithelial cells resulted in oxytocin increasing the PGE to PGF ratio.

Cyclooxygenase-1 (PTGS-1) and COX-2 (PTGS-2) are the main cyclooxygenase in mammalian cells, with COX-1 being constitutively expressed in most tissues, whereas COX-2 is upregulated in response to inflammatory stimuli. The promoter region of COX-2 gene contains binding sites for several transcription factors, including NF-κB (MacKenzie et al., 2013). Lipopolysaccharide leads to the activation of NF-κB (Lu et al., 2008), which may explain the increased expression of COX-2 mRNA and PG secretion by endometrial cell after LPS challenge (Herath et al., 2006).

Effects of Endotoxin on Blood Cortisol and Haptoglobin Concentration

Under stressful situations, the activity of the sympathetic nervous system is changed, leading to an increase in cardiovascular function and a release of adrenal catecholamines (Jansen et al., 1995). This phenomenon is known as the “fight or flight” response, which is essential for host survival.

During stressful situations such as endotoxemia, glucocorticoids play an important role in maintaining homeostasis because it prevents an exacerbated inflammatory response, which is essential for survival. Cortisol release is controlled by the hypothalamic-pituitary-adrenal (HPA) axis. According to Beishuizen and Thijs (2003), adrenalectomized mice had increased mortality after LPS, IL-1 or TNFα challenge, but adrenalectomized mice survived these challenges when glucocorticoid was administered. Glucocorticoids modulate the inflammatory response by suppressing pro-inflammatory cytokines such as IL-1, IL-6, and TNFα, and by upregulating anti-inflammatory cytokines such as IL-4 and IL-10.

Glucocorticoids also reduce expression of adhesion molecules on endothelial cells through indirect and direct mechanisms (Barnes, 1998). Glucocorticoids indirectly reduce expression of adhesion molecules because they inhibit secretion of cytokines such as IL-1β and TNFα, which are known to induce expression of adhesion molecules on endothelial cells. On the other hand, glucocorticoids inhibit gene transcription for ICAM-1 and E-selectin, directly reducing expression of adhesion molecules (Cronstein et al., 1992).

The exact mechanism through which LPS and cytokines stimulates HPA axis is complex and still not completely understood. Lipopolysaccharides increase the levels of TNFα, IL-1 and IL-6 that stimulates secretion of corticotropin-releasing hormone by the hypothalamus, which stimulates secretion of adrenocorticotropic hormone (**ACTH**) by the pituitary. Adrenocorticotropic hormone stimulates the synthesis and secretion of glucocorticoids from the adrenal cortex. A direct effect of LPS on pituitary and adrenal is

also speculated because some studies demonstrated that LPS may stimulate corticosterone secretion after removal of the medial hypothalamus (Beishuizen and Thijs, 2003).

Vakharia and Hinson (2005) demonstrated that LPS stimulates cortisol secretion by human adrenocortical cell line NCI-H295R, a line of adrenocortical tumor cells. Because these cells express TLR2 and TLR4, it was suggested by the authors that these receptors mediate the effects of LPS in adrenal cells.

Among the systemic consequences of endotoxemia, in response to increased secretion of pro-inflammatory cytokines, is the production by the liver of acute-phase proteins (**APP**). In ruminants, haptoglobin (**Hp**) is a major APP and has been suggested as a diagnostic marker for diseases such as mastitis and respiratory diseases (Hiss et al., 2004).

Haptoglobin is a plasma protein synthesized by hepatocytes that binds to free hemoglobin to prevent oxidative damage. Once bound to Hp, haemoglobin loses its oxidizing ability. Haptoglobin-haemoglobin complex is removed from the circulation through endocytosis after recognition by the receptor CD163 expressed by monocytes and macrophages (Carter and Worwood, 2007).

Haptoglobin is a member of the APP family because its production is strongly increased by pro-inflammatory cytokines such as IL-6. Haptoglobin role on the immune system is still unclear, but it is speculated that Hp acts to regulate the immune responses with an anti-inflammatory activity (Huntoon et al., 2008).

In summary, cows with vaginal purulent discharge have impaired reproductive

performance but there is no product available in the United Stated for treatment of this disease. This dissertation describes and discusses experiments that evaluated the effects of treating cows with vaginal purulent discharge intrauterinally with an immunomodulator (*E. coli* LPS) on inflammatory/immune responses, uterine health, and reproductive performance. The main hypothesis of these experiments was that intrauterine treatment with *E. coli* LPS would induce local and systemic inflammatory responses leading to resolution of vaginal purulent discharge and improvements in reproductive performance of lactating dairy cows.

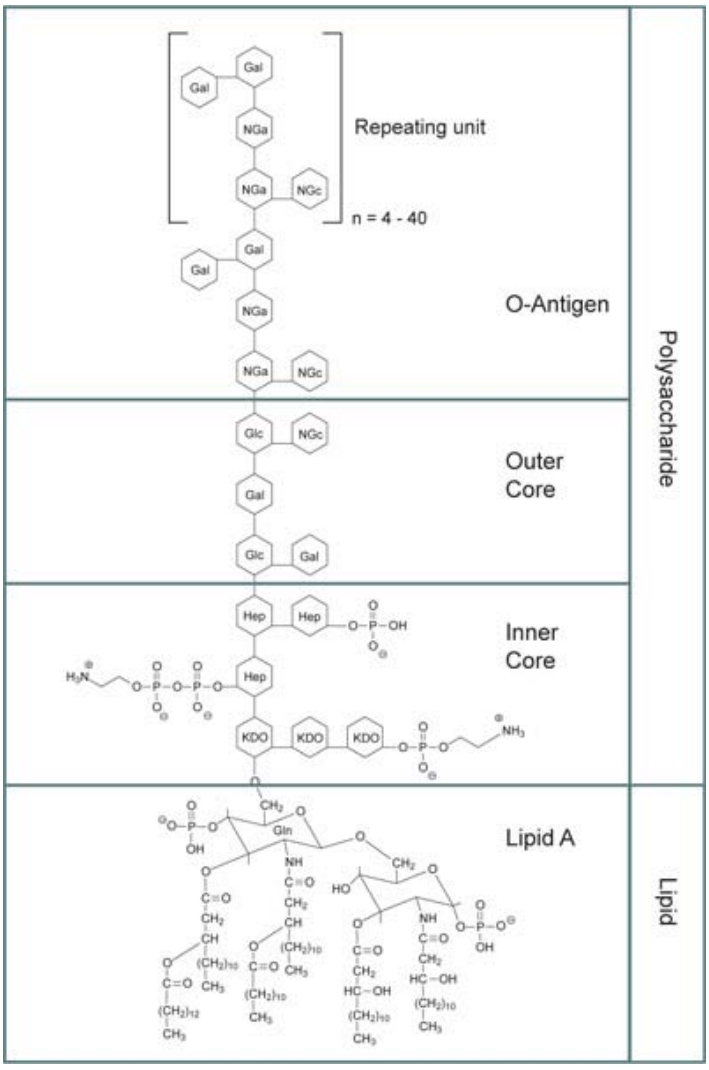

Figure 1. General structure for bacterial lipopolysaccharides. Abbreviations: KDO: 3-deoxy-α-D-mannooctulosonic acid; Hep: Heptulose (ketoheptose); NGa: Galactosamine; NGc: Glucosamine. Source: Retrieved May 10, 2013, from the Sigma-Aldrich Co website <http://www.sigmaaldrich.com/technical-documents/articles/biology/glycobiology/lipopolysaccharides.html>.

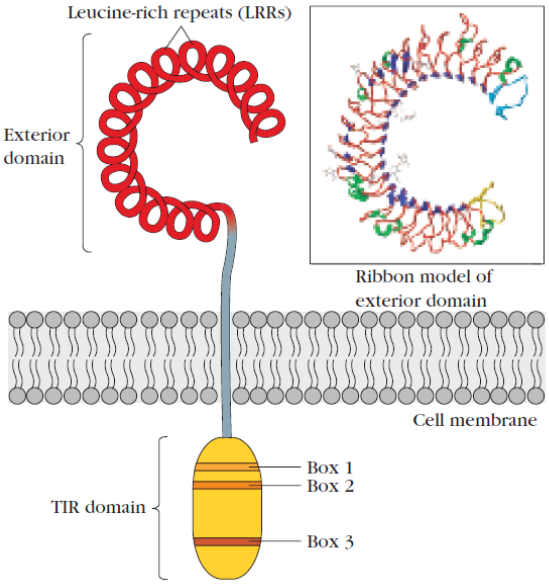

Figure 2. Structure of a Toll-like receptor (TLR). Taken from: Kindt, T. J., R. A. Goldsby, B. A. Osborne, and J. Kuby. 2007. Kuby immunology, Figure 3-10. WH Freeman & Company.

Chapter 2

The Manuscript

Effects of intra-uterine infusion with *Escherichia coli* lipopolysaccharide on endometrial mRNA gene expression, peripheral polymorphonuclear leukocyte activity, hemogram parameters, and cortisol, haptoglobin and progesterone concentrations of Jersey cows diagnosed with vaginal purulent discharge

OVERVIEW

The objectives of the current experiment were to investigate the effects of intra-uterine treatment of cows with vaginal purulent discharge (VPD) with lipopolysaccharide (LPS) from *Escherichia coli* on endometrial mRNA expression of genes related to inflammatory responses; peripheral polymorphonuclear leukocytes (PMNL) function; hematological parameters; and circulating concentrations of cortisol, haptoglobin and progesterone. Jersey cows (n = 3,084) were examined for vaginal purulent discharge at 31 ± 3 d postpartum using the Metricheck device. Cows with at least 50% of pus in the exudate retrieved from the cranial vagina were characterized as positive for VPD. At examination, 2,664 cows were determined to be healthy, 310 cows were determined positive for VPD (10.1%), but only 267 cows with VPD were used in this experiment. Cows with VPD were balanced at 31 ± 3 d postpartum for lactation number, body condition score (BCS), VPD score (mild, moderate, severe), and weekly average milk

yield, and randomly assigned to the control treatment [intrauterine infusion of 20 mL of phosphate-buffered saline (PBS); n = 87] or to receive 20 mL of PBS containing 150 μg of LPS (LPS150; n = 91) or 300 μg of LPS (LPS300; n = 89) of *E. coli* LPS (*E. coli* serotype 026:B6, containing 10,000 endotoxin units per mg of LPS; Sigma-Aldrich, St. Louis, MO, USA). Lipopolysaccharide treatment occurred 24 h after diagnosis of VPD and enrollment. A sub-sample of cows had their endometrium biopsied 6 h after infusion and another sub-sample of cows had their endometrium biopsied 24 h after infusion to evaluate the effect of treatment on endometrial mRNA expression of genes related to inflammatory responses. Peripheral blood polymorphonuclear leukocyte expression of adhesion molecules [L-selectin and β2-Integrins (CD18)], phagocytosis and oxidative burst were evaluated immediately before treatment (0 h) and again at 2 and 6 h after treatment. Blood samples on 0, 2, 6, 24, and 48 h were used for hemogram; on 0, 2, 4, 6, and 24 h for determination of cortisol; and on 0, 24, 48 and 168 h for determination of haptoglobin and progesterone (P4) concentrations. There was no effect of treatment on endometrial mRNA expression of adhesion molecules [endothelial leukocyte adhesion molecule (E-selectin), intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)], cytokines (tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-8 and IL-10) and Toll-like receptor-4 (TLR-4) in biopsies collected at 6 or 24 h after treatment. There was no effect of treatment on PMNL expression of L-selectin, but geometric mean fluorescence intensity (GMFI) of PMNL expressing CD18 was higher for LPS150 cows (5143.77 ± 248.17) than control cows (4208.69 ± 243.92) and tended to be higher in LPS150 than LPS300 cows (4505.66 ± 243.40). Cows enrolled in the

LPS300 treatment had greater percentage of PMNL positive for both, phagocytosis and oxidative burst (67.0% ± 1.46), compared to control (62.0% ± 1.50) and LPS150 (62.2% ± 1.46) cows. There was no significant effect of treatment on hemogram parameters and circulating concentrations of cortisol, haptoglobin and progesterone (P4). These observations suggest that intrauterine infusion of *E. coli* LPS moderately stimulates circulating PMNL phagocytosis and expression of CD18 but it has no effect on endometrial expression of mRNA of genes related to the inflammatory response. Further research is needed to better understand the mechanisms by which LPS could act as an immunomodulator for treatment of VPD.

INTRODUCTION

Reproductive failure, the most important reason for involuntary culling of dairy cows (NAHMS, 2007), greatly affects profitability of the cattle industry. Endometritis, an inflammation of the inner layer of the uterus that is usually associated with bacterial infection, affects approximately 20% of postpartum dairy cows (Galvão, 2012) and compromises establishment of new pregnancies, reduces milk production and increases culling (Sheldon et al., 2009). Percentage of endometritic cows pregnant after first postpartum AI may be reduced by as much as one third (Gilbert et al., 2005). Commonly isolated bacterium from the uterus of cows with endometritis include *Arcanobacterium pyogenes*, *Escherichia coli*, *Prevotella melaninogenicus*, *Fusobacterium spp., Streptococcus spp.*, *Staphylococcus spp.*, *Pasteurella multocida*, *Clostridium spp.*, and *Bacillus licheniformis* (Williams et al., 2005). Immune deficiency is one of the most

important factors that predispose lactating dairy cows to uterine diseases (LeBlanc, 2008). In cows that develop metritis and endometritis, phagocytic capacity of neutrophils isolated from peripheral blood increases from parturition to the third week postpartum, but the number and phagocytic ability of neutrophils recovered from the uterus declines from the first to the third week postpartum (Hammon et al., 2006; Mateus et al., 2002). The cause of such asynchrony between peripheral blood and uterine neutrophil activity has not been determined, but it is possible that the reduced counts of bacteria in the uterus and increased concentrations of progesterone (**P4**) may reduce the chemotactic stimulus to the uterus (Mateus et al., 2002). There are no products currently approved in the United States for treatment of endometritis. Intrauterine treatment of endometritic cows using 500 mg of cephapirin benzathine in 19 g ointment base (Metricure®, Intervet, Boxmeer, The Netherlands) is approved in Canada, Europe and New Zealand (Galvão, 2012), and has been reported to improve reproductive performance of cows with clinical (LeBlanc et al., 2002) and subclinical (Kasimanickam et al., 2005) endometritis. However, cost of treatment associated with possible development of microbial resistance to antibiotics, makes interesting the idea of development of non-antimicrobial treatments that effectively improves fertility. Furthermore, intra-uterine antibacterial therapy may inhibit phagocytic activity of neutrophil in the uterus (Oxender and Seguin, 1976; Massera et al., 1980), which may compromise recovery from uterine infections.

Lipopolysaccharide present in the outer membrane of gram-negative bacteria cell wall plays important role in pathogen recognition and triggering the subsequent inflammatory response (Mogensen, 2009) and has been used as an immunomodulator. In

mammals, the physiological recognition of bacterial LPS occurs trough the Toll-like receptor-4 (**TLR-4**) in association with myeloid differentiation factor 2 (**MD-2**) (Park et al., 2009). After LPS binding to the complex TLR4-MD-2, TLR4 Toll-interleukin 1 receptor (**TIR**) domain plays a critical role in signal transduction through protein-protein interactions among TLR-4 and five adaptor proteins, leading to the activation of transcription factors such as nuclear factor-κB (**NF-κB**) and members of the interferon-regulatory factor family (Lu et al., 2008; O'Neill and Bowie, 2007). Upon NF-κB activation, many cytokines such as tumor necrosis factor-α (**TNF-α**), IL-1, IL-6 and IL-8 are produced (Wright, 1999), stimulating both innate and adaptive immunity (Li and Verma, 2002; Schroder et al., 2004). Interleukin-1 and TNF-α are functionally related pro-inflammatory cytokines that mediate many of the consequences of sepsis including fever, vasodilatation and stimulation of other cytokines (Marsh et al., 1994). Interleukin-6 is a cytokine with both pro and anti-inflammatory properties. Acting as pro-inflammatory cytokine, IL-6 in association with IL-1β or TNF-α stimulates the production of various chemokines leading to the attraction of neutrophils in the initial phase of inflammation. However, by 24-48 h after initial infiltration, neutrophils are replaced by monocytes and T cells in order to prevent tissue damage from accumulation of proteases and reactive oxygen species produced by neutrophils. Within this context, IL-6 induces neutrophil apoptosis contributing to resolution of the acute neutrophil infiltration (Scheller et al., 2011). Interleukin-6 also upregulates expression of adhesion molecules on endothelial cells and leukocytes (Scheller et al., 2011), induces acute-phase protein production by the liver (e.g. haptoglobin), and stimulates hematopoiesis (Kishimoto et al., 1992).

Furthermore, TNF-α, IL-1 and IL-6 stimulate secretion of glucocorticoids from the adrenal cortex. Increasing concentrations of cortisol prevents an exacerbated inflammatory response, which is essential for host survival (Beishuizen and Thijs, 2003). Interleukin-8, member of the chemokine family, plays an important role guiding leukocytes to sites of inflammation, increases neutrophil expression of integrin adhesion molecules LFA-1 (CD11aCD18) and MAC1 (CD11bCD18) and induces neutrophils to release lysosomal enzymes (Harada et al., 1994). Interleukin-10 possesses immunosuppressive and anti-inflammatory properties because it prevents antigen-specific T cell activation and down regulates the production of pro-inflammatory cytokines and chemokines in many cell types (de Vries, 1995). Moreover, adhesion molecules expressed on endometrial cells [endothelial leukocyte adhesion molecule (**E-selectin**), intracellular adhesion molecule-1 (**ICAM-1**) and vascular cell adhesion molecule (**VCAM-1**)] play a critical role in leukocyte-endothelium interactions allowing leukocytes to migrate to inflamed tissues (Kindt et al., 2007). Nuclear-factor κB also stimulates cyclooxygenase-2 (**COX-2**; MacKenzie et al., 2013), resulting in increased prostaglandins being produced following LPS stimulus, which might interfere with the luteolysis process (Herath et al., 2006).

The hypothesis of the current study is that intra-uterine treatment of cows with vaginal purulent discharge (VPD) with *E. coli* LPS will modulate endometrial mRNA expression of genes related to the inflammatory response, will increase PMNL activity (expression of adhesion molecules, phagocytosis and oxidative burst), will increase blood cortisol and haptoglobin concentrations, and, in cows with a corpus luteum (**CL**) present

in the ovary, will stimulate luteolysis. Our objectives were to investigate the effects of intra-uterine treatment of endometritic cows with LPS from *E. coli* on endometrial mRNA expression of genes related to the inflammatory response, circulating PMNL function, hematological parameters, and circulating concentrations of cortisol, haptoglobin and P4.

MATERIAL AND METHODS

Cows and Treatments

The University of Minnesota Institutional Animal Care and Use Committee reviewed and approved all procedures. Jersey lactating cows (n = 3,084) from a free-stall dairy located in Nicollet, MN were evaluated for vaginal purulent discharge (**VPD**) at 31 ± 3 d postpartum using the Metricheck device (Simcro, New Zealand; McDougall et al., 2007) from December 2010 to May 2012. Cows with $\geq$ 50% of pus in the retrieved vaginal exudate were considered positive for VPD. At examination, 2,664 cows were determined to be healthy, and there were 420 (13.6%) cows with $\geq$ 50% of pus in the retrieved exudate. These cows were examined by palpation per rectum of the uterine contents and 110 cows were determined to have large amounts of fluid accumulated in the uterus and therefore were not treated in the study. The remaining 310 cows were designated positive for VPD (10.1%), but only 267 cows were assigned to treatments because in each enrollment day cows were enrolled in the order that they found up to a maximum of 9 cows enrolled per day. Cows with VPD (n = 267) were balanced at 31 ± 3 d postpartum for lactation number, BCS, VPD score (mild, moderate, severe), and

weekly average milk yield, and randomly assigned to the control treatment (intrauterine infusion of 20 mL of PBS (n = 87) or to receive an intrauterine infusion of 20 mL of PBS containing 150 μg of *E. coli* LPS (**LPS150**; n = 91) or to receive an intrauterine infusion of 20 mL of PBS containing 300 μg of *E. coli* LPS (**LPS300**; n = 89). Lipopolysaccharide was from *E. coli* serotype 026:B6 containing 10,000 endotoxin units per mg of LPS (Sigma-Aldrich, St. Louis, MO, USA). Lipopolysaccharide treatment occurred 24 h after diagnosis of VPD and enrollment.

Vaginal Purulent Discharge Score

Scores for VPD were given based on visually observation of retrieved exudate, which was placed on a black plastic plate to improve visualization. Scores were given according to the following classification: mild (retrieved exudate contained approximately 50 to 60% of pus); moderate (retrieved exudate contained approximately 60 to 90% of pus); and severe (retrieved exudate contained approximately 90 to 100% pus).

General Cow Management

All cows were milked thrice daily and were fed a fresh TMR twice daily. With respect to the reproductive management, voluntary waiting period was 50 DIM and cows were inseminated when observed in estrus after the end of the voluntary waiting period. Cows were presynchronized with three injections of prostaglandin (**PG**) $F_{2\alpha}$ given 41 ± 3, 55 ± 3 and 69 ± 3 DIM. Cows not inseminated in estrus were enrolled in the Ovsynch56 protocol (GnRH, 7 d later $PGF_{2\alpha}$, 56 h later GnRH) 81 ± 3 DIM and were inseminated at

fixed time 91 ± 3 DIM. As for resynchronization of the estrous cycle of non-pregnant cows, cows enrolled in the study from December 2010 to September 2011 received the first GnRH of the Ovsynch56 protocol at 31 ± 3 d after insemination and if diagnosed not pregnant 38 ± 3 d after AI, received a $PGF_{2\alpha}$ injection at non-pregnancy diagnosis, GnRH 56 h later, and TAI 16h later. Cows enrolled in the study from February 2012 to May 2012 were examined by ultrasound (5 MHz, Ibex Lite; E. I. Medical Imaging, Loveland, CO) for pregnancy diagnosis at 31 ± 3 d after AI and non-pregnant cows were enrolled in one of two resynchronization protocols depending on ovarian structures present in the ovaries at the time of non-pregnancy diagnosis. Cows that had a CL $\geq$ 20 mm in diameter received an injection of $PGF_{2\alpha,}$ and if not inseminated in estrus in the following 12 d were enrolled in the Ovsynch56. Cows that at non-pregnancy diagnosis did not have a CL $\geq$ 20 mm in diameter received a GnRH injection at non-pregnant diagnosis and were enrolled in the Ovsynch56 protocol 7 d later. Cows diagnosed pregnant at 31 ± 3 or 38 ± 3 d after AI were re-examined 66 ± 3 and 184 ± 3 d after AI.

Endometrial Biopsies

A sub-sample of cows (control, n = 16; LPS150, n = 17; LPS300, n = 17) had their endometrium biopsied 6 h after infusion and another sub-sample of cows (control, n = 18; LPS150, n = 17; LPS300, n = 14) had their endometrium biopsied 24 h after infusion. Biopsies were collected as described by Galvão et al. (2011). Briefly, before collecting biopsy samples, an epidural anesthesia (4 mL of 2% lidocaine) was performed. Samples were collected using a Hauptner biopsy instrument that was protected with a sanitary chemise (IMV Technologies, L'Aigle, France), which was broken immediately

before the biopsy tool was passed through the cervical os. Biopsies (weighed between 100 and 200 mg) were collected from the base of the larger horn (whenever it was possible to distinguish), which was presumed to be the previously gravid horn, and placed in 2 ml microcentrifuge tubes containing 1 mL of a commercial sample buffer (RNAlater®, Ambion-Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) and frozen at -80°C until RNA isolation.

RNA Isolation and Purification

RNA isolation and purification was done as described by Cerri et al. (2012). Briefly, endometrial samples were removed from the -80°C freezer and kept on shaved ice until extraction. The RNA was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and purified using PureLink™ RNA Mini Kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. The RNA concentration, purity and integrity were determined using the Agilent 2100 Bioanalyzer Nano Drop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Samples were aliquoted and stored at -80°C until amplification.

Quantitative, Real-Time, Reverse Transcription-PCR

Quantitative RT-PCR was performed using Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Life Technologies Corporation) as described previously (Ozawa et al., 2013). Briefly, RNA samples (50 ng/reaction) were incubated with RNase-free DNase I (New England BioLabs, Ipswich, MA) for 15 min at 37°C, heat denatured (75°C for 10 min), and then reverse transcribed using High Capacity cDNA

Reverse Transcription Kit and random hexamers (Applied Biosystems) and amplified with SYBR Green PCR Master Mix reagent (Applied Biosystems) under PCR normal conditions (40–50 cycles of 95°C for 15 s and 60°C for 1 min). Primers used in the current experiment were designed using Primer Express v2.0 (Applied Biosystems) and are shown in Table 1. Mitochondrial ribosomal protein S15 (**MRPS15**) was used as housekeeping gene as this has been shown to be the most stable normalizer gene in postpartum cows (Chapwanya et al., 2009). A standard curve was built for each gene of interest by doing four 1:5 dilutions starting with 100 ng/μL of cDNA solution. The efficiency of the amplification was calculated, and only primer sets with efficiency > 90% were used. Specificity of amplification of each primer set was examined by dissociation curve analysis, and only sets with one single peak were used. All reactions were done in triplicate, and triplicate samples with Ct coefficient of variation > 1% were repeated. A no template control was run for each primer used on every 96-well plate. Fold change (**n-fold**) in gene expression was calculated using the relative quantitation method (2^{-ddCt}) in which MRPS15 was used as endogenous control and the average delta Ct of samples collected from cows that received intrauterine infusion with PBS as the calibrator for each sample.

Peripheral Polymorphonuclear Cell Activity

Ex vivo innate immune parameters were evaluated from a sub-group of 25 cows per treatment at 0, 2, and 6 h relative to treatment as described by Hulbert et al. (2011). Samples were collected into heparinized evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Expression of leukocyte adhesion molecule-1 (**L-**

selectin) and CD18 by peripheral PMNL was determined by indirect immunofluorescence staining. Briefly, the assay consisted of incubating 200 µL of whole blood at 4°C for 30 min with 5 µg/mL of anti-bovine CD62L (DU1-29, VMRD Inc., Pullman, WA) monoclonal antibody produced in mouse or 2.5 µg/mL of anti-bovine CD18 (BAQ30A, VMRD Inc., Pullman, WA, USA) monoclonal antibody produced in mouse. Prior to the incubation of cells with an anti-mouse IgG-FITC secondary polyclonal antibody (AbD Serotec, Raleigh, NC, USA) diluted 1:400 in PBS solution (Sigma-Aldrich, St. Louis, MO, USA), erythrocytes were lysed with hypotonic PBS solution. After washing the cells with PBS solution, samples were analyzed by flow cytometry. Blood from non-diseased cows were used as positive and negative controls in all assays. Negative controls consisted of incubating 200 µL of PBS solution instead of the monoclonal antibody. Phagocytic and oxidative burst activity of peripheral PMNL were determined upon challenge with enteropathogenic bacteria (*E. coli* 0118:H8) as previously described by Hulbert et al. (2011). Briefly, the assay to determine phagocytosis and oxidative burst consisted of incubating 200 µL of whole blood with 100 µM of dihydrorhodamine 123 (Molecular Probes, Invitrogen, USA), an oxidative-sensitive indicator, and 40 µL of fluorescently labeled bacteria (10^9 cfu/mL) at 38.5°C for 15 min, with surface bacteria fluorescence removed using the Trypan Blue Solution (0.4%; Sigma-Aldrich, St. Louis, MO, USA). After washing with milliQ water to remove excess dye, erythrocytes were lysed by the addition of hyper-concentrated PBS solution (Sigma-Aldrich, St. Louis, MO, USA). Lastly, the cells were resuspended in PBS solution for immediate flow cytometry analysis. Blood from non-diseased cows were

used as positive and negative controls. Unlabeled bacteria were used as negative controls for the phagocytosis assay and samples that received no dihydrorhodamine 123 served as negative controls for the oxidative burst assay. All flow cytometry data were collected on a BD FACSCANTO II (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo 7.6.4 software (Tree Star Inc., San Carlos, CA, USA). The PMNL population was identified on basis of forward and side scattered properties. After strictly gating the PMNL population, data from 3 parameters were collected for analysis: forward scatter, side scatter, and log fluorescence. Intensity of phagocytosis, oxidative burst, and expression of CD18 and L-selectin molecules by PMNL was expressed in geometric mean fluorescence intensity (**GMFI**). Intensity of phagocytosis and expression of adhesion molecules were an indirect indication of the number of phagocytised bacteria and antibody against CD62L or CD18 adhere to PMNL, respectively. Oxidative burst intensity was an indirect indication of the amount of reactive oxygen species produced via oxidation of dihydrorhodamine 123. Furthermore, percentages of PMNL positive for phagocytosis, oxidative burst, and expression of CD18 and L-selectin molecules were calculated.

Hemogram

Blood samples on 0, 2, 6, 24, and 48 h after treatment were collected from a sub-group of cows (control, n = 38; LPS150, n = 40; LPS300, n = 41) for hemogram. Samples collected into evacuated tubes with K3 EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) were analysed using a Vet Scan HM2 (Abaxis, Union City,

CA). Complete blood count was performed but only data referent to counts of leukocytes, lymphocytes, monocytes and granulocytes are reported.

Cortisol, Haptoglobin and Progesterone Assays

For determination of concentrations of cortisol, haptoglobin and P4 blood was sampled (7 mL) from the median coccygeal vein or artery using evacuated tubes and immediately placed in ice and transported to the laboratory within 5 h of collection. Blood tubes were centrifuged at 2,000 × g for 15 min for serum or plasma separation. Serum and plasma samples from individual cows were frozen at -25°C until analyzed.

For determination of cortisol concentration, blood samples from a sub-group of cows (control, n = 43; LPS150, n = 45; LPS300, n = 42) were collected into evacuated tubes without anticoagulant (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) at 0, 2, 4, 6, and 24 h relative to treatment, and serum was analysed using a solid-phase radioimmunoassay kit (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The intra- and inter-assay coefficients of variation (**CV**) were 9.32% and 3.59%, respectively.

Haptoglobin concentration was determined by a colorimetric procedure (Hulbert et al., 2011) using a plate reader (Spectramax 340; Molecular Devices, Sunnyvale, CA) to measure absorbance. Samples were collected into evacuated tubes without anti-coagulant (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) 0, 1, 2, and 7 d after treatment from all cows. The intra-assay and inter-assay CV were 4.64 and 3.55%, respectively.

Blood samples for determination of P4 concentration were collected from all cows on d 0, 1, 2, and 7 after treatment into evacuated tubes containing EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). All samples collected on d 0 were analyzed for P4 concentration and cows with P4 ≥ 1 ng/mL were classified as having a CL present in the ovary at the time of treatment. Cows with P4 ≥ 1 ng/mL on d 0 had samples collected on d 1, 2 and 7 analyzed for P4 concentration to determine if LPS treatment stimulated luteolysis. Progesterone concentration in plasma was determined by RIA (Coat-a-Count Progesterone, Siemens Medical Solutions Diagnostics, Los Angeles, CA) and the intra-assay and inter-assay CV were 7.28 and 6.46%, respectively.

Statistical Analysis

The design of the experiment was complete randomized with cows balanced for lactation number, BCS, VPD score, and weekly average milk yield. All statistical analyses were conducted using SAS (SAS Institute Inc., Cary, NC).

For endometrial mRNA gene expression, UNIVARIATE procedure revealed that n-fold was not normally distributed; therefore, ANOVA was performed on the delta delta Ct (**ddCt**) values using GLM procedure. Fold change values were used to present the data.

Data of peripheral PMNL function, hemogram, and concentrations of cortisol, haptoglobin and P4 were analyzed by ANOVA for repeated measures using MIXED procedure. All models included treatment, time, treatment by time interaction and the baseline value as a covariate (0 h or 0 d). Baseline data were analyzed by ANOVA using

GLM procedure. Because of the possible effects of endometrial biopsy on cortisol and haptoglobin concentrations, whether or not the cow was biopsied and time that biopsy was collected (6 or 24 h after treatment) were included in the model (0 = no biopsy; 6 = biopsy 6 h; 24 = biopsy 24 h). Cortisol, haptoglobin and P4 data were not normally distributed and therefore statistical analysis was performed using log to the base 10 of original values, but data are presented as back transformed values. Finally, proportion of cows assumed to have a CL present in the ovary (P4 > 1 ng/mL) at moment of treatment, and proportion of cows that had luteolysis following treatment were analyzed by Fisher's Exact Test using FREQ procedure in samples collected at d 0 and on d 1, 2 and 7 after treatment.

Statistical significance was defined as $P \leq 0.05$ and statistical tendencies as $0.05 < P \leq 0.10$.

RESULTS

Endometrial Biopsies

Endometrial mRNA expression of adhesion molecules (E-selectin, ICAM-1 and VCAM-1), cytokines (TNF-α, IL-1β, IL-6, IL-8 and IL-10), and TLR-4 at 6 h (Figure 3) and 24 h (Figure 4) after treatment were not ($P \geq 0.25$) affected by treatment.

Peripheral Blood Polymorphonuclear Cell Activity

Percentage of PMNL positive for expression of L-selectin ($P = 0.74$) and intensity of expression of L-selectin ($P = 0.80$) were not affected by treatment (Table 2).

Percentage of PMNL positive for expression of CD18 was not affected by treatment (P = 0.76). Geometric mean fluorescence intensity of expression of CD18 was higher for LPS150 than control cows (P = 0.01) and tended to be higher for LPS150 than LPS300 cows (P = 0.07), but there was no difference between control and LPS300 cows (P = 0.39; Table 2).

Percentage of PMNL positive for phagocytosis and oxidative burst were affected by treatment (P = 0.03; Table 2, Figure 5), which was higher in LPS300 cows (67.00%) than in control (61.95%; P = 0.02) and LPS150 cows (62.23%; P = 0.02). Moreover, there was no effect of treatment in GMFI for phagocytosis (P = 0.35) and oxidative burst (P = 0.64) in PMNL positive for both phagocytosis and oxidative burst (Table 2).

Hemogram

Selected results of hemogram analysis are shown in Table 3. Treatment had no effect on counts of leukocytes (P = 0.63), granulocytes (P = 0.56), lymphocytes (P = 0.21) and monocytes (P = 0.35). Granulocyte to lymphocyte ratio was also not (P = 0.25) affected by treatment (Figure 6).

Cortisol, Haptoglobin and Progesterone Assays

Cortisol (P = 0.72; Figure 7) and haptoglobin (P = 0.89; Figure 8) concentrations were not affected by treatment. Furthermore, the interactions between treatment and sample did not affect cortisol (P = 0.12) and haptoglobin (P = 0.26) concentrations.

Proportion of cows with P4 $\geq$ 1.0 ng/mL 0 h relative to treatment was not different (P = 0.55) among treatments [control = 51.72% (45/87); LPS150 = 43.96%

(40/91); LPS300 = 44.94% (40/89)]. Moreover, within cows with P4 $\geq$ 1.0 ng/mL at moment of treatment, the proportion of cows that had luteolysis P4 $\leq$ 1.0 ng/mL at d 1 [*P* = 0.42; control = 13.33% (6/45); LPS150 = 12.50% (5/40); LPS300 = 22.50% (9/40)], d 2 [*P* = 0.36; control = 11.11% (5/45); LPS150 = 15.00% (6/40); LPS300 = 22.50% (9/40)] and d 7 [*P* = 0.95; control = 51.11% (23/45); LPS150 = 51.28% (20/39); LPS300 = 47.50% (19/40)] were also not affected by treatment. Furthermore, there was no (*P* = 0.33) effect of treatment on P4 concentration following treatment (Figure 9). Lower concentrations of P4 at d 7 were expected because cows received a prostaglandin injection at 41 ± 3 d postpartum as part of the reproductive program.

DISCUSSION

It was expected that treatment of cows with VPD with LPS would alter the pattern of endometrial mRNA expression of genes for adhesion molecules, cytokines and TLR-4 compared to control cows, but this was not observed in the present study. One possibility that would explain this outcome is that by 6 and 24 h after treatment mRNA gene expression that was initially upregulated was already downregulated. In a study conducted by Schmitz et al. (2004) mRNA expression of TNF-α in mammary biopsy samples of quarters intramammarily infused with LPS peaked at 3 h after treatment, and by 12 h, TNF-α mRNA expression was near baseline values. Another possibility for no differences in endometrial mRNA expression of genes related to inflammatory response in the present study could be attributed to the population used in the study, which were cows believed to have clinical endometritis according to Sheldon et al. (2006) disease

definition. Endometritic cows are known to have the uterus colonized by both gram-negative and gram-positive aerobes and anaerobes (Azawi, 2008), and LPS is the major component of the outer membrane of gram-negative bacteria cell wall. Galvão et al. (2011) evaluated endometrium mRNA gene expression of pro-inflammatory (TNFα, IL-1β, IL-6 and IL-8) and anti-inflammatory (IL-10) cytokines in cows that developed endometritis and in healthy cows at weeks 1, 3, 5 and 7 after calving. They observed that mRNA gene expression for pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) was increased in endometritic cows on weeks 5 and/or 7 after calving compared with healthy cows. Thus, one limitation of the present study is that uterine biopsies were not collected from healthy cows as it is possible that the lack of differences in expression of endometrial mRNA for pro-inflammatory genes between control and LPS treated cows may have been because all cows enrolled already had an up-regulation of these genes. Furthermore, endometrial mRNA expression of adhesion molecules 6 and 24 h after intrauterine infusions with PBS or LPS was not different. E-selectin is a cell adhesion molecule member of the selectin family that through interactions with its ligand expressed on the surface of leukocytes (L-selectin) is responsible for the initial stickiness of leukocytes to endothelium, an early step in the recruitment of these cells to a site of injury or inflammation (Springer, 1990). In addition, surface glycoprotein ICAM-1 expressed by endometrial cells through interaction with its ligand CD11aCD18 expressed by leukocytes promotes leukocytes' firm adhesion and diapedeses during leukocyte emigration from the bloodstream to extravascular tissues (Carlos and Harlan, 1994).

Although no differences among treatments were observed regarding PMNL expression of L-selectin, LPS150 cows had greater intensity of expression of CD18 than control cows. This increased expression of CD18 in LPS150 cows could result in improved migratory capacity of PMNL from the blood to the uterus. Endometritic cows treated with 150 μg of LPS had numerically higher counts of PMNL in uterine cytology than control and LPS300 cows in samples collected 24 and 48 h after intra-uterine infusion (Moraes et al., 2012a).

Polymorphonuclear leukocyte phagocytosis and oxidative burst were partially affected by treatment. Non-specific phagocytosis by neutrophils is one of the most important lines of defense against establishment of uterine infections (LeBlanc, 2008). In the present study, LPS300 cows had greater percentage of PMNL positive for phagocytosis and oxidative burst than control and LPS150 cows. It was expected that LPS treated cows would have greater oxidative burst than control cows, as activation of cell NADPH oxidase, an important enzyme responsible for generating large amounts of superoxide during the oxidative burst process, can be induced by LPS, by lipoproteins, or by cytokines such as IFN-γ, IL-1β, and IL-8 (Droge, 2002). Moreover, no alterations on hemogram parameters were observed as consequence of LPS intra-uterine infusion. Intramammary challenge with 1,000 μg of LPS resulted in leukopenia 6 to 12 h after treatment (NADIR 6 h after treatment) and a peak concentration of immature neutrophils in the circulation 6 h after LPS treatment (Mehrzad et al., 2001). However, leukocytosis was observed from 24 to 48 h after intramammary infusion with LPS (Mehrzad et al., 2001). Furthermore, circulating concentration of leukocytes was greater in LPS treated

cows compared with control cows by 216 h after treatment. In the present study, granulocyte to lymphocyte ratio was numerically higher for LPS treated cows than control cows, and granulocyte to lymphocyte ratio peaked 6 h after treatment and was still numerically higher by 48 h.

Blood cortisol concentrations were not affected by treatment. Lipopolysaccharide increases concentrations of TNFα, IL-1 and IL-6, which stimulate the secretion of corticotropin-releasing hormone by the hypothalamus resulting in secretion of adrenocorticotropic hormone (**ACTH**) by the pituitary and glucocorticoids by the adrenal cortex (Beishuizen and Thijs, 2003). The lack of differences among treatments regarding cortisol concentration may result from handling induced stress during sample collection since all cows had a significant rise in cortisol concentration 4 to 6 h after intra-uterine infusions. Concentrations of haptoglobin were not different among treatments. Similarly, concentrations of the acute phase protein alpha-1 acid glycoprotein was not different between Holstein heifers receiving intrauterine infusion of 3 μg/kg of LPS (dose superior than higher dose used in the present study) or PBS (Williams et al., 2008). On the other hand, Hiss et al. (2004) reported significant increase in circulating haptoglobin concentrations 12 h after administration of 100 μg of LPS in the jugular vein of lactating dairy cows. Plasmatic concentration of LPS following challenge was not measured in the present study, but we speculate that the increase in haptoglobin concentration observed by Hiss et al. (2004) might be due to higher LPS concentration because of intravenous administration.

The lack of differences in percentages of cows with progesterone concentration ≥ 1 ng/mL at moment of intra-uterine infusion is important because progesterone is immunosuppressant (Siiteri et al., 1977; Hansen, 2013). Expression of mRNA for pro-inflammatory chemokines and cytokines (CXCL5, IL-1β, and IL-8) are reduced during the diestrus compared with the proestrus (Fischer et al., 2010). Moreover, progesterone inhibits *in vitro* endometrial and epithelial production of PGE and PGF series in response to LPS challenge (Herath et al., 2006) and PG play a key role in the regulation of the inflammatory response (Ricciotti and FitzGerald, 2011). Among cows with CL present in the ovary in the current study, cows treated with LPS had numerically lower concentrations of P4 at d 1 and 2 after treatment. Moreover, the proportion of cows with P4 < 1 ng/mL at d 1 and 2 were numerically higher in LPS300 cows. Thus, we speculate that some cows might have initiated the process of luteolysis due increases in PGF secretion, and that this one week with lower progesterone concentrations before cows received the PGF2α of the synchronization protocol, might have contributed for better immune function in the uterus. Intrauterine treatment of cows with VPD with 150 μg or 300 μg of LPS improved pregnancy at first postpartum artificial insemination compared to cows with VPD treated with just PBS, and pregnancy by 200 days postpartum was also greater for cows with VPD that were treated with 150 μg of LPS than cows with VPD treated with PBS (Moraes et al., 2013a). Thus, we believe that the sum of findings PMNL function, numerical differences in circulating leukocytes and reduction in progesterone concentration in cows with CL present in the ovary following LPS treatment, might have

contributed to improved reproductive performance that we observed (Moraes et al., 2013a).

CONCLUSIONS

Treatment of cows with VPD with intrauterine infusion of *E. coli* LPS moderately stimulated circulating PMNL phagocytosis and expression of CD18 molecule, but had no significant effect on endometrial mRNA expression of inflammatory genes, hemogram parameters, and cortisol, haptoglobin or progesterone concentrations. The sum of findings of the present study contributed to understanding pathways by which LPS could be acting as an immunomodulator, as data from our group suggest improved reproductive performance in endometritic cows upon intrauterine treatment with LPS. However, these findings are relatively weak to explain the big difference in reproductive performance that we observed, therefore further research is needed in order to better understand the inflammatory and immune mechanisms of *E. coli* LPS in the uterus of dairy cows diagnosed with VPD.

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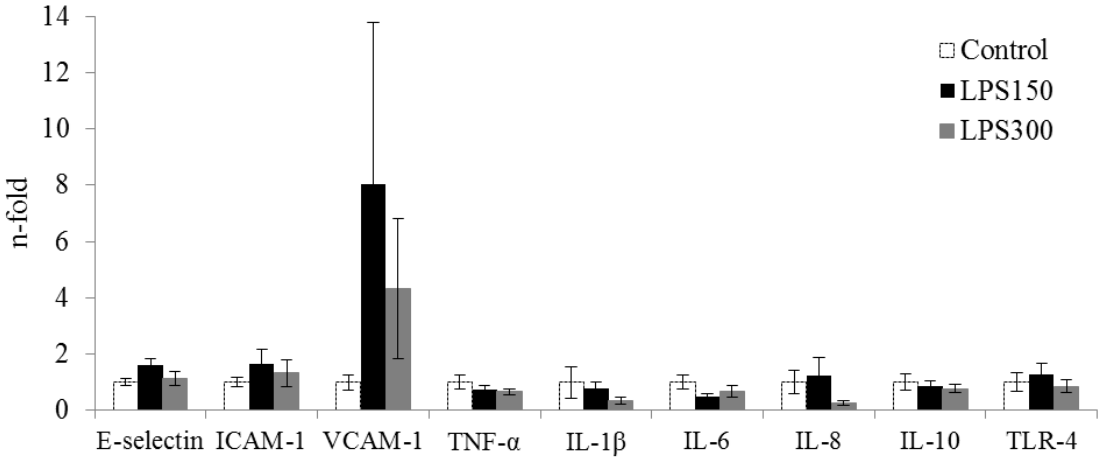

Figure 3. Effect of treatment on endometrium mRNA expression of adhesion molecules, cytokines and Toll-like receptor 4 (TLR-4) in endometrium biopsies collected 6 h after infusion. Treatments: Control (n = 16) - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 (n = 17) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 μg of *E. coli* LPS as treatment; LPS300 (n = 17) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 μg of *E. coli* LPS as treatment. Treatment had no effect on endometrial mRNA expression of E-selectin ($P = 0.28$), ICAM-1 ($P = 0.43$), VCAM-1 ($P = 0.38$), TNF-α ($P = 0.61$), IL-1β ($P = 0.45$), IL-6 ($P = 0.79$), IL-8 ($P = 0.33$), IL-10 ($P = 0.83$) and TLR-4 ($P = 0.25$).

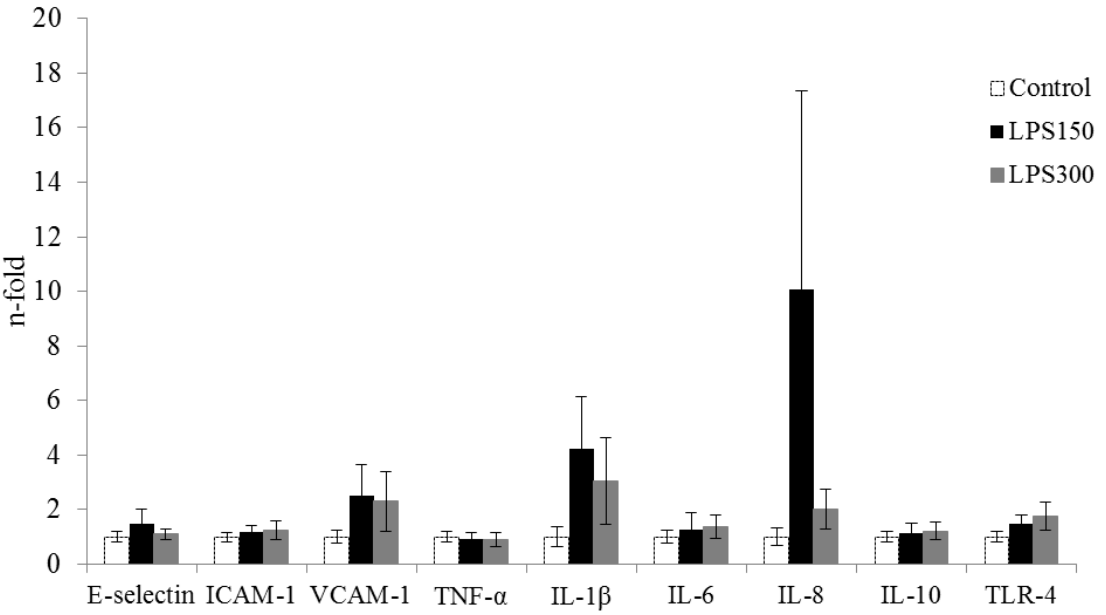

Figure 4. Effect of treatment on endometrium mRNA expression of adhesion molecules, cytokines and Toll-like receptor 4 (TLR-4) in endometrium biopsies collected 24 h after infusion. Treatments: Control (n = 18) - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 (n = 17) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of E. coli LPS as treatment; LPS300 (n = 14) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of E. coli LPS as treatment. Treatment had no effect on endometrial mRNA expression of E-selectin ($P = 0.60$), ICAM-1 ($P = 0.77$), VCAM-1 ($P = 0.73$), TNF-α ($P = 0.44$), IL-1β ($P = 0.37$), IL-6 ($P = 0.48$), IL-8 ($P = 0.70$), IL-10 ($P = 0.91$) and TLR-4 ($P = 0.40$).

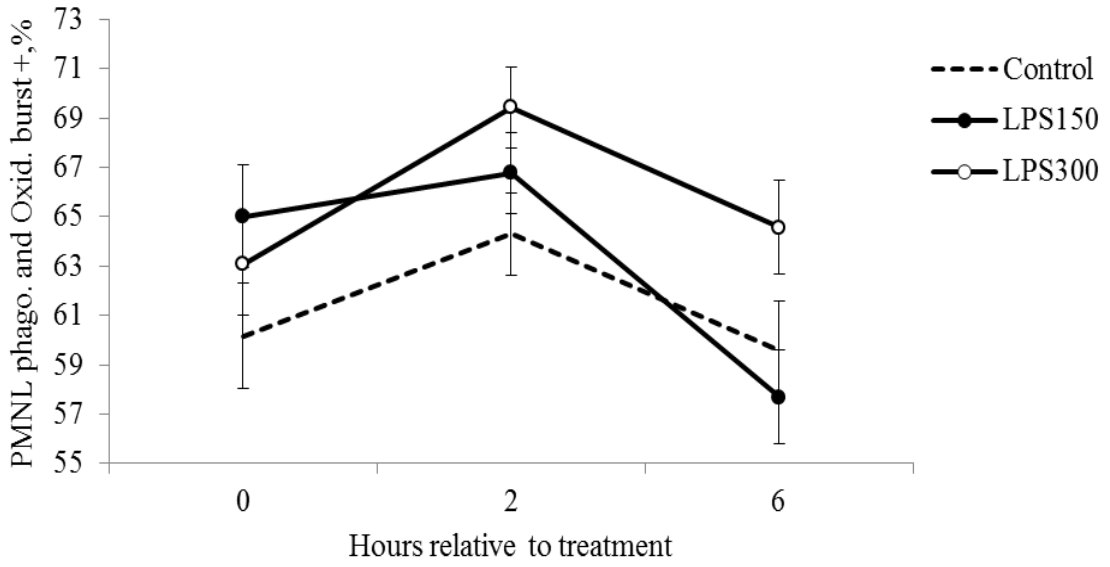

Figure 5. Effect of treatment on percentage of peripheral blood PMNL positive for phagocytosis and oxidative burst. Treatments: Control (n = 25) - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 (n = 25) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of *E. coli* LPS as treatment; LPS300 (n = 25) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of *E. coli* LPS as treatment. No difference among treatments (P = 0.27) in percentage of PMNL positive for phagocytosis and oxidative burst were observed in the sample collected before treatment, but it was higher (P = 0.03) in LPS300 cows than control cows at 2 h; higher in 300LPS (P = 0.01) than 150LPS cows at 6 h; and tended (P = 0.07) to be higher in 300LPS than control cows at 6 h. Furthermore, there was no interaction of treatment by sample (P = 0.23).

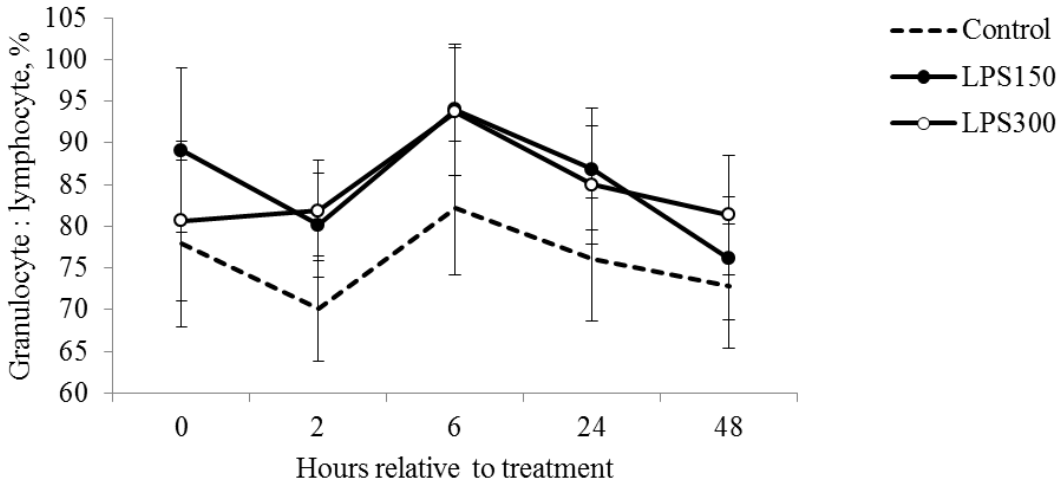

Figure 6. Effect of treatment on granulocyte to lymphocyte ratio. Treatments: Control (n = 38) - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 (n = 40) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of *E. coli* LPS as treatment; LPS300 (n = 41) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of *E. coli* LPS as treatment. There was no effect of treatment (P = 0.25) on granulocyte to the lymphocyte ratio, and there was no interaction (P = 0.99) of treatment by sample.

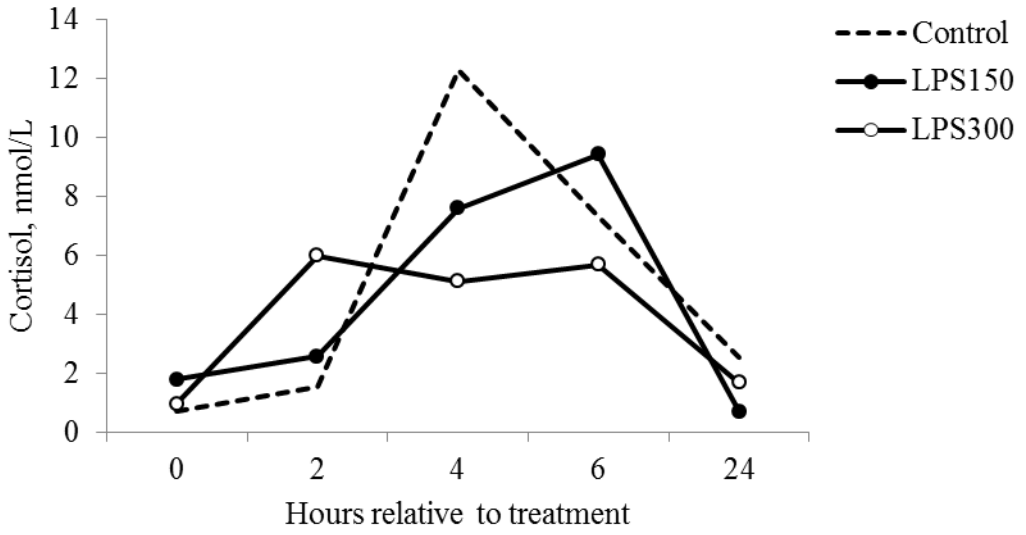

Figure 7. Effect of treatment on blood cortisol concentration. Cortisol concentrations are expressed in back transformed values from logarithm to the base 10 of original results. Treatments: Control (n = 43) - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 (n = 45) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of *E. coli* LPS as treatment; LPS300 (n = 42) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of *E. coli* LPS as treatment. There were no differences among treatments on cortisol concentrations at baseline (P = 0.48) or thereafter (P = 0.72) and there was no interaction (P = 0.12) of treatment by sample.

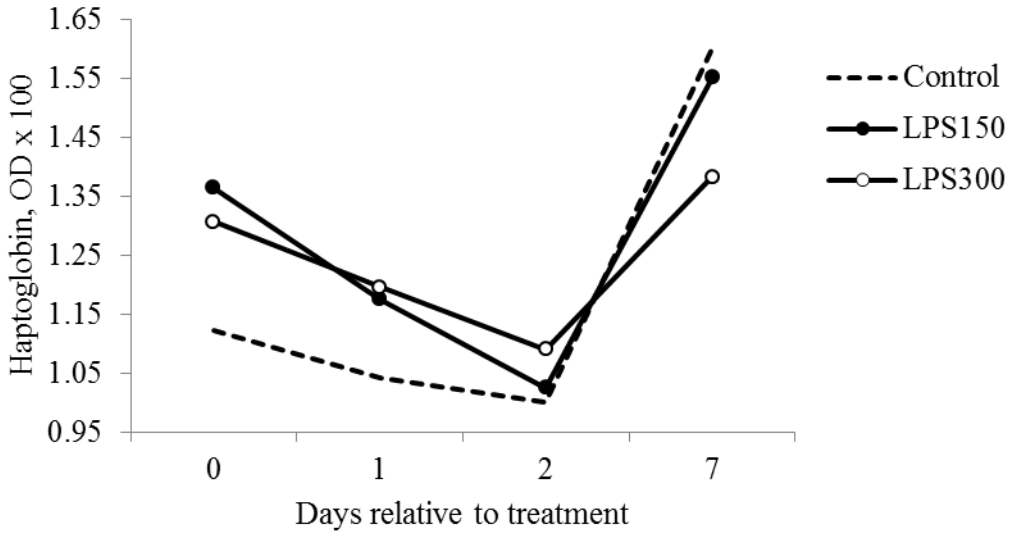

Figure 8. Effect of treatment on blood haptoglobin concentration. Haptoglobin concentrations are expressed in back transformed values from logarithm to the base 10 of original results. Treatments: Control (n = 87) - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 (n = 91) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of *E. coli* LPS as treatment; LPS300 (n = 89) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of *E. coli* LPS as treatment. There was no difference among treatments on haptoglobin concentrations at baseline (P = 0.12) or thereafter (P = 0.89), and there was no interaction of treatment by sample (P = 0.26).

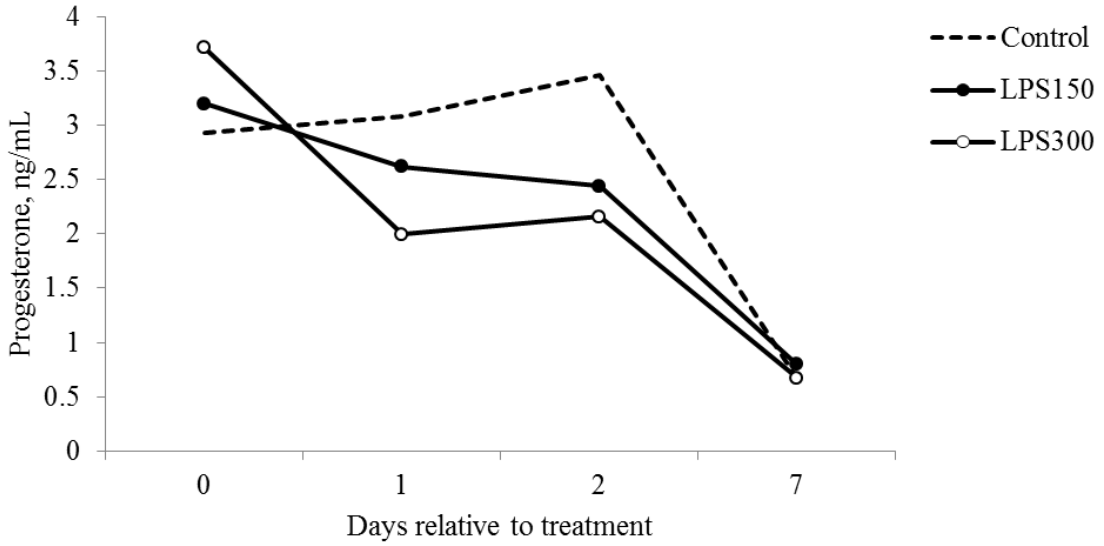

Figure 9. Effect of treatment on progesterone (P4) concentration of cows that had P4 $\geq$ 1 ng/mL in the sample collected right before treatment. Progesterone concentrations are expressed in back transformed values from logarithm to the base 10 of original results. Treatments: Control (n = 87) - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 (n = 91) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of *E. coli* LPS as treatment; LPS300 (n = 89) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of *E. coli* LPS as treatment. There was no statistical difference (P = 0.33) in progesterone concentration following treatment in cows with CL present in the ovary (P4 > 1 ng/mL) at moment of infusion, and there was no interaction of treatment by sample (P = 0.68).

Table 1. Gene used for quantitative reverse transcription-PCR.

Gene	GenBank ID	Primer	Sequence (5' to 3')
MRPS15	NM_001192201.1	Forward	AGATGACCCGCCCCCTTCCA
		Reverse	GGGAGCTGGTGTCCTTCGGGT
E-selectin	NM_174181.2	Forward	AGGCCACTCGGTGCATGTCC
		Reverse	CAACCTGGGTTGGCCCCTGC
ICAM-1	NM_174348.2	Forward	GGGCCTGCCGTGCTCCTTAC
		Reverse	AACAGCCCTCCCCTCCGGTC
VCAM-1	NM_174484.1	Forward	AGGGCTCAGTTAGAGGATGCGGG
		Reverse	CAAGGAAGCCTGAACCCCCAGT
TNF-α	NM_173966.2	Forward	ACACACCCCCGAGAGACAGCC
		Reverse	TGGGGGCCGATTACCCCGAA
IL-1β	NM_174093.1	Forward	GCTTCAGGCAGGTGGTGTCGG
		Reverse	GCACGGGTGCGTCACACAGA
IL-6	NM_173923.2	Forward	ATTCGCTGTCTCCCTGGGGCT
		Reverse	AGTCTGCCTGGGGTGGTGTCAT
IL-8	NM_173925.2	Forward	TGGCTGTTGCTCTCTTGGCAGC
		Reverse	CCTGCACAACCTTCTGCACCCA
IL-10	NM_174088.1	Forward	GAGAGTCTTCAGTGAGCTCCAAGAG
		Reverse	GCATCTTCGTTGTCATGTAGGTTT

Table 2. Effect of intra-uterine infusion with LPS or PBS on PMNL expression of adhesion molecules (L-selectin and CD18), phagocytosis and oxidative burst 0, 2, and 6 h after infusion.

						P-value
Items	Control[1]	LPS150[1]	LPS300[1]	TRT	Sample	TRT x Sample
PMNL expressing L-selectin, %	51.94 ± 3.60	55.94 ± 3.66	53.81 ± 3.61	0.74	0.35	0.58
Intensity of L-selectin expression, GMFI	930.75 ± 53.09	904.70 ± 53.89	880.86 ± 53.28	0.80	0.33	0.51
PMNL expressing CD18, %	95.87 ± 1.57	97.45 ± 1.58	97.05 ± 1.56	0.76	0.33	0.15
Intensity of CD18 expression, GMFI	4208.69 ± 243.92[a]	5143.77 ± 248.17[b,A]	4505.66 ± 243.40[B]	0.03	0.54	0.35
PMNL phago. and oxid. burst positive, %	61.95 ± 1.50[a]	62.23 ± 1.46[a]	67.00 ± 1.46[b]	0.03	< 0.01	0.23
Intensity of phagocytosis, GMFI	3268.49 ± 98.63	3067.90 ± 96.37	3189.82 ± 96.81	0.35	0.01	0.20
Intensity of oxidative burst, GMFI	7746.60 ± 471.62	7143.07 ± 459.55	7601.14 ± 462.79	0.64	0.22	0.22

[a,b] Within a row, numbers with different lowercase letters (a,b) are different ($P < 0.05$).

[A,B] Within a row, numbers with different uppercase letters (A,B) tended ($0.05 < P \leq 0.10$) to be different.

[1] Treatments: Control (n = 25) - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 (n = 25) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 μg of *E. coli* LPS as treatment; LPS300 (n = 25) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 μg of *E. coli* LPS as treatment.

Table 3. Effect of treatment on hemogram parameters.

Parameters, 10^9/L ± SEM[1]	Treatment			*P* - value		
	Control[2]	LPS150[2]	LPS300[2]	TRT	Sample	TRT x Sample
White blood cells	10.64 ± 0.22	10.93 ± 0.21	10.75 ± 0.21	0.63	< 0.01	0.77
Granulocytes	3.75 ± 0.18	3.85 ± 0.18	4.02 ± 0.18	0.56	< 0.01	0.58
Lymphocytes	6.57 ± 0.24	6.77 ± 0.23	6.20 ± 0.23	0.21	0.04	0.59
Monocytes	0.32 ± 0.05	0.22 ± 0.05	0.26 ± 0.05	0.35	0.72	0.72
Granulocyte:lymphocyte	75.27 ± 4.77	84.27 ± 4.71	85.48 ± 4.59	0.25	0.04	0.99

[2] Treatments: Control (n = 38) - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 (n = 40) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of *E. coli* LPS as treatment; LPS300 (n = 41) - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of *E. coli* LPS as treatment.

CHAPTER 3

The manuscript

Effects of intra-uterine infusion of *Escherichia. coli* lipopolysaccharide on uterine health, resolution of vaginal purulent discharge and reproductive performance of lactating dairy cows

OVERVIEW

The objectives of the current experiment were to evaluate the effects of intra-uterine infusion of cows diagnosed with vaginal purulent discharge (VPD) with *Escherichia coli* lipopolysaccharide (LPS) on intrauterine cell population, uterine health and reproductive performance of lactating dairy cows. Jersey cows (n = 3,084) were examined for vaginal purulent discharge at 31 ± 3 d postpartum using the Metricheck device (Simcro, New Zealand; McDougall et al., 2007). Cows with the retrieved exudate containing at least 50% of pus were characterized positive for VPD. At examination, 2,664 cows were negative for VPD and were used as positive controls. There were 420 (13.6%) cows with > 50% of pus in the retrieved exudate, and from that, 159 VPD cows were selected to be treated in the current study. Selected cows were balanced at 31 ± 3 d postpartum for lactation number, body condition score, VPD score (mild, moderate, severe), and weekly average milk yield, and were randomly assigned to receive intrauterine infusion of 20 mL of phosphate-buffered saline (PBS; control, n = 51), or 20 mL of PBS containing 150 µg (LPS150, n = 54) or 300 µg (LPS300, n = 54) of *Escherichia coli* LPS (*E. coli* serotype 026:B6, containing 10,000 endotoxin units per mg

of LPS; Sigma-Aldrich, St. Louis, MO, USA). Incidences of dystocia, twin calving, male calf, retained fetal membrane and metritis were recorded for individual cows. Cows were examined with the Metricheck at 7 and 28 d after treatment to evaluate effect of treatment on resolution of vaginal purulent discharge. Uterine cytology was performed before treatment and again on d 1, 2 and 7 after treatment to evaluate the effects of treatment on intrauterine cell population. Reproductive parameters were recorded up to 200 days postpartum. Cows diagnosed with VPD at 31 ± 3 d postpartum had greater incidence of dystocia (control = 12.2%, LPS150 = 17.0%, LPS300 = 17.0% and healthy = 3.4%), retained placenta (control = 13.7%, LPS150 = 16.7%, LPS300 = 9.3% and healthy = 1.7%) and metritis (control = 25.5%, LPS150 = 24.1%, LPS300 = 20.4% and healthy = 4.0%) after calving than healthy cows, but there was no difference in incidence of these events among cows with VPD only. Incidences of twining (control = 4.1%, LPS150 = 3.8%, LPS300 = 3.8% and healthy = 1.9%) and male calf (control = 44.9%, LPS150 = 49.1%, LPS300 = 43.4% and healthy = 48.0%) were not different between cows with VPD and healthy cows. Percentage of cows with VPD at 7 (control = 58.0%, LPS150 = 55.8%, LPS300 = 67.9%) and 28 d (control = 10.6%, LPS150 = 13.7%, LPS300 = 11.8%) after treatment were not affected by treatment. No differences in percentage of polymorphonuclear cells in uterine cytology were observed on d 0 or thereafter. The percentage of cows pregnant 31 ± 3 d after first postpartum artificial insemination (AI) was lower for control cows (23.9%) than for LPS150 (42.0%), LPS300 (43.8%) and healthy (49.9%) cows, but it was similar between LPS treated and healthy cows. Furthermore, likelihood of pregnancy 66 days after first postpartum AI was not different

among LPS150 (36.0%), LPS300 (35.4%) and healthy cows (46.1%), but control cows were less likely (21.7%) to be pregnant at 66 ± 3 days after first AI than healthy cows. Moreover, pregnancy 31 ± 3 d after the second postpartum AI was higher for healthy cows (45.0%) than control (22.2%) and LPS300 (25.0%), but it was not different between healthy and LPS150 (40.6%) cows, or among cows with VPD only. Similarly, likelihood of pregnancy at 66 ± 3 days after second postpartum AI was higher for healthy cows (40.6%) compared to control (22.2%) and LPS 300 (16.0%) cows, but it was not different between healthy and LPS150 cows (38.7%) or among cows with VPD only. Pregnancy loss after first and second insemination was not affected by treatment. Thus, according to the current experiment, intrauterine treatment of cows with VPD using 150 μg of *E. coli* LPS solution improved reproductive performance, but further research is needed to elucidate the mechanism by which LPS treatment improved fertility.

INTRODUCTION

Profitability of dairy farms is greatly affected by the reproductive performance of the herd because it affects milk production, number of replacements produced, and voluntary and involuntary culling (Britt, 1985). In the first 2 weeks postpartum, cows are at high risk for uterine infections caused primarily by *Escherichia coli* and *Trueperella pyogenes* (Sheldon et al., 2009), concomitant with *Prevotella melaninogenicus*, *Fusobacterium spp., Streptococcus spp.*, *Staphylococcus spp.*, *Pasteurella multocida*, *Clostridium spp.*, and *Bacillus licheniformis* (Williams et al., 2005). Insufficient intake of energy, protein, vitamins and minerals, and dramatic changes in concentrations of

steroidal hormones predispose postpartum lactating dairy cows to immunodeficiency and uterine infections (Huzzey et al., 2007; LeBlanc, 2008). Uterine bacterial contamination occurs in approximately 80 to 90 % of dairy cows during the first two weeks after parturition (Sheldon et al., 2009) and 10 to 20% of them develop endometritis (Potter et al., 2010), whereas approximately 30% develop sub-clinical endometritis (Galvão, 2012). Cows that have dystocia, twining, retained fetal membranes, stillbirth, abortion, metritis, male offspring, and ketosis are at higher risk for endometritis (Galvão, 2012).

Endometritis, an inflammation of the endometrium, has a negative effect on profitability because of consequent infertility, increased culling for reproductive failure, reduced milk yield, and treatment cost (Sheldon et al., 2009). Clinical endometritis is characterized in cattle as the presence of a purulent (> 50% pus) uterine discharge detectable in the vagina after 21 DIM or a mucopurulent discharge (approximately 50% pus and 50% mucus) detectable in the vagina after 26 DIM without any systemic symptoms (Sheldon et al., 2006). Currently, there are no products approved in the USA for intrauterine treatment of endometritic cows. Intrauterine treatment of endometritic cows using 500 mg of cephapirin benzathine in 19 g ointment base (Metricure®, Intervet, Boxmeer, The Netherlands) is approved in Canada, Europe and New Zealand (Galvão, 2012), and has been reported to improve reproductive performance of cows with clinical (LeBlanc et al., 2002) and subclinical (Kasimanickam et al., 2005) endometritis. However, cost of treatment associated with possible development of microbial resistance to antibiotics, makes interesting the idea of development non-antimicrobial treatments that effectively improves fertility. Furthermore, intra-uterine antibacterial therapy may

inhibit phagocytic activity of neutrophil in the uterus (Oxender and Seguin, 1976; Massera et al., 1980), which may compromise recovery from uterine infections.

Lipopolysaccharide, a constituent of the outer membrane of gram-negative bacteria cell, plays an important role in pathogen recognition and triggering inflammatory response (Mogensen, 2009). In mammals, recognition of LPS occurs trough the toll-like receptor (**TLR**) 4 in association with myeloid differentiation factor 2 (**MD-2**; Park et al., 2009). After LPS binds to the complex TLR4-MD-2, TLR4 Toll-interleukin 1 receptor (**TIR**) domain plays a critical role in signal transduction and leads to the activation of transcription factors such as nuclear factor-κB and members of the interferon-regulatory factor family (O'Neill and Bowie, 2007; Lu et al., 2008). The activation of these two factors regulates the transcription of a variety of genes, including cytokines and chemokines, that control the innate and adaptive immunity (Li and Verma, 2002; Schroder et al., 2004). Further, among several systemic and local reactions stimulated by many cytokines and chemokines, these proteins stimulate leukocytes migration to inflamed tissues. Interleukin-8 for instance, is an important cytokine that act guiding leukocytes to the site of inflammation (Harada et al., 1994). Interleukin-1β and TNFα are known to induce expression of many adhesion molecules on endothelial cells (Cronstein et al., 1992) that through interaction with adhesion molecules expressed by leukocytes, allow them to migrate from the blood vessel to the inflamed tissues (Albelda et al., 1994). Moreover, LPS has also the ability to stimulate neutrophils killing ability. Through TLR-4, LPS stimulates NADPH oxidase activation leading to production of large amounts of superoxide (Huang et al., 2009).

Intra-uterine infusion of endometritic cows with 100 μg of *E. coli* LPS resulted in elimination of uterine bacterial infection in 75% of treated the cows and increased intra-uterine total leukocyte count, proportion of PMNL, and proportion of live PMNL in the uterus (Singh et al., 2000). Further, 8 of the 12 cows treated with LPS conceived, whereas only 1 of the 12 untreated controls conceived (Singh et al., 2000). These are promising results, but the small number of cows used, the low milk yield (< 22 lb/d), and differences in management systems (India vs. USA) precludes extrapolating these results to dairy cows in the USA.

Therefore, the hypothesis of the current study was that intra-uterine treatment of cows with vaginal purulent discharge (**VPD**) with *E. coli* LPS increases number of PMNL in the uterus, resolves VPD, improves pregnancy per AI after first and second postpartum AI, and improves pregnancy rates up to 200 DIM. The objectives of the present study were to evaluate the effects of intra-uterine infusion of cows with VPD with *E. coli* LPS on intrauterine cell population, uterine health, and reproductive performance of lactating dairy cows.

MATERIAL AND METHODS

The University of Minnesota Institutional Animal Care and Use Committee reviewed and approved all procedures performed in the study. From December 2010 to May 2012, lactating Jersey cows (n = 3,084) from a free-stall dairy located in Nicollet (MN) were examined for VPD at 31 ± 3 DIM using the Metricheck device (Simcro, New Zealand; McDougall et al., 2007). Cows with the retrieved exudate containing at least

50% of pus were characterized positive for VPD. At examination, 2,664 cows were determined to be healthy and were used as a positive control. Four hundred and twenty (13.6%) cows were positive VPD. These cows were examined by palpation per rectum of the uterine contents and 110 cows were determined to have large amounts of fluid accumulated in the uterus and therefore were not treated in this experiment. From the remaining 310 cows with VPD (10.1%), 267 were assigned to treatments (Moraes and Chebel, unpublished data), from which 108 were assigned to have the uterus biopsied. Because the objective of the present study was to evaluate the effects of LPS treatment on uterine health and reproductive performance, and because cows that have the uterus biopsied are expected to have lower fertility, data from the remaining 159 cows with VPD were used in this experiment. These cows were balanced for lactation number, body condition score, VPD score (mild, moderate, severe), and weekly average milk yield, and were randomly assigned to receive intrauterine infusion of 20 mL of **PBS** (**control**, n = 51), or 20 mL of PBS containing 150 µg (**LPS150**, n = 54) or 300 µg (**LPS300**, n = 54) of *E. coli* LPS (*E. coli* serotype 026:B6, containing 10,000 endotoxin units per mg of LPS; Sigma-Aldrich, St. Louis, MO, USA). Lipopolysaccharide treatment occurred 24 h after diagnosis of VPD and enrollment. The doses of LPS used for treatments were defined based on a pilot study that demonstrated that intra-uterine infusions of 100, 150, 200, 250, and 450 µg of LPS diluted in 20 mL of PBS did not pose a risk to the health of cows (Moraes and Chebel, unpublished data). Further, data from uterine cytology collected 20 h after treatment demonstrated that the correlation between dose of LPS and

PMNL isolated was quadratic with maximum response obtained with doses > 200 μg (Moraes and Chebel, unpublished data).

Vaginal Purulent Discharge Score

Scores for VPD were given based on visual observation of retrieved exudate, which was placed on a black plastic plate to improve visualization. Scores were given according to the following classification: mild (retrieved exudate contained approximately 50 to 60% of pus); moderate (retrieved exudate contained approximately 60 to 90% of pus); and severe (retrieved exudate contained approximately 90 to 100% pus).

Postpartum Problems

Occurrence of dystocia, twin calving, male calf, retained fetal membrane and metritis were recorded for all cows examined for VPD using on farm software (DairyComp305, Valley Agricultural Software, Tulare, CA) and were collected for individual cows.

Calving ease score was recorded for all cows' right after calving. Calving ease score was defined as follows: 1 (cow that calved by itself but in the close-up pen rather than in the maternity pen); 2 (cow that calved by itself in the maternity pen); 3 (cow that was unable to calve by itself and therefore required assistance, e.g. correcting calf presentation, position and/or posture); 4 (calving that required fetotomy); and 5 (calving that required cesarean section). Cows with calving ease score of 3 to 5 were classified as having dystocia.

Multiparous and primiparous cows were kept in two different pens from parturition to 15 DIM. In these pens, cows were evaluated daily by the herd veterinarian and trained farm personnel for common postpartum diseases, including retained fetal membrane and metritis. An event for retained fetal membrane was recorded for cows that retained their placenta for at least 24 h after calving. All cows were examined daily from 4 to 12 DIM, and cows with enlarged uterus containing watery, fetid, reddish-brownish uterine discharge were classified as having metritis. Cows diagnosed with metritis received a ceftiofur hydrochloride sterile suspension (Excenel RTU EZ; Pfizer Animal Health, Madison, NJ) for 4 consecutive days at a dose of 2.2 mg/kg of body weight. Cows with abnormal uterine discharge on d 5 after treatment were retreated with the same dose of Excenel for additional 3 consecutive days.

Uterine Health and Resolution of Vaginal Purulent Discharge

To evaluate the effects of intrauterine infusion with LPS on uterine health, treated cows were examined with the Metricheck 7 and 28 d after treatment. Resolution of VPD was defined when there was less than 50 % of pus in the retrieved exudate.

Effect of Treatment on Intrauterine Cell Population

To determine the effect of treatment on intrauterine cell population, all treated cows had uterine cytology performed before treatment and again on d 1, 2 and 7 after treatment. Cytology was performed using the cytobrush (Cytobrush Plus®, Cooper Surgical, Inc) technique. After sample collection the cytobrush was rolled onto a clean microscope glass slide, which was stained with modified Wright-Giemsa stain (Protocol-

Hema3, Biochemical Sciences, Swedesboro, New Jersey, USA). Slides were evaluated twice, and at each examination 100 cells excluding erythrocytes were counted under 400x magnification by one examiner who was blinded to treatments and interval between sample collection and treatment.

Reproductive Parameters

Voluntary waiting period was 50 DIM and cows were inseminated when observed in estrus after the end of the voluntary waiting period. Cows were presynchronized with three injections of prostaglandin (**PG**) $F_{2\alpha}$ given 41 ± 3, 55 ± 3 and 69 ± 3 DIM. Cows not inseminated in estrus were enrolled in the Ovsynch56 protocol (GnRH, 7 d later $PGF_{2\alpha}$, 56 h later GnRH) 81 ± 3 DIM and were inseminated at fixed time 91 ± 3 DIM. As for resynchronization of the estrous cycle of non-pregnant cows, cows enrolled in the study from December 2010 to September 2011 received the first GnRH of the Ovsynch56 protocol at 31 ± 3 d after insemination and if diagnosed not pregnant 38 ± 3 d after AI received a $PGF_{2\alpha}$ injection at non-pregnancy diagnosis, GnRH 56 h later, and TAI 16h later. Cows enrolled in the study from February 2012 to May 2012 were examined by ultrasound (5 MHz, Ibex Lite; E. I. Medical Imaging, Loveland, CO) for pregnancy diagnosis 31 ± 3 d after AI and non-pregnant cows were enrolled in one of two resynchronization protocols depending on ovarian structures present in the ovaries at the time of non-pregnancy diagnosis. Cows that had a corpus luteum $\geq$ 20 mm in diameter received an injection of $PGF_{2\alpha,}$ and if not inseminated in estrus in the following 12 d were enrolled in the Ovsynch56. Cows that at non-pregnancy diagnosis did not have a corpus luteum $\geq$ 20 mm in diameter received a GnRH injection at non-pregnant diagnosis and

were enrolled in the Ovsynch56 protocol 7 d later. Cows diagnosed pregnant at 31 ± 3 or 38 ± 3 d after AI were re-examined 66 ± 3 and 184 ± 3 d after AI. Pregnancy and pregnancy loss after first and second inseminations and rate at which cows became pregnant up to 200 DIM were calculated.

Production Parameters

Cows were milked thrice daily and all cows received recombinant bST (500 mg of Posilac; Elanco Animal Health, Greenfield, IN) every 10 d starting 57 ± 3 DIM. Monthly, milk yield, milk fat and protein content, and somatic cell count were recorded for individual cows during the official Dairy Herd Improvement Association test. Data regarding milk yield, milk fat and protein content, and somatic cell count were collected from calving to 200 d postpartum. Energy corrected milk was calculated for each cow using the formula (Orth, 1992):

ECM (kg) = [(kg milk) x 0.327] + [(kg fat) x 12.95] + [(kg protein) x 7.2].

Statistical Analysis

All statistical analyses were conducted using SAS (SAS Institute Inc., Cary, NC). Variable treatment was forced in all models.

Descriptive statistics for incidence of male calf, twin calving, dystocia, retained fetal membranes and metritis were analyzed by logistic regression using LOGISTIC procedure, and the proportions of cows experiencing each of these events were determined using FREQ procedure. The distribution of parity (primiparous vs. multiparous) was analyzed by Fisher's Exact Test using FREQ procedure.

Incidence of VPD 7 and 28 d after treatment was analyzed by logistic regression using LOGISTIC procedure. Models included just treatment as independent variable because there was no difference in occurrence of all parameters evaluated in the descriptive statistics among cows with VPD. Proportion of cows with VPD at each examination was computed using FREQ procedure.

Baseline intrauterine PMNL counts and percentage were analyzed by ANOVA using the GLM procedure, and PMNL counts and percentage at d 1, 2 and 7 after treatment were analyzed by ANOVA for repeated measures using MIXED procedure. Model included treatment, time of sample collection, treatment by time interaction, and the baseline values as a covariate. Furthermore, percentage of cows with > 10% of PMNC in uterine cytology was analyzed by logistic regression using LOGISTIC procedure.

Milk yield and components data were analyzed by ANOVA for repeated measures using MIXED procedure. Variables offered to all models included treatment, twins, dystocia, retained fetal membranes, metritis, test number (relative to days postpartum sample was collected), interaction of treatment and test number, and group (relative to the weekly cohorts of cows examined for VPD). Variable group was included in the model for analyses of milk production and reproductive performance as two different synchronization protocols were used during the study time. Variable was retained if $P < 0.2$.

Pregnancy at first and second postpartum AI and pregnancy loss between 31 ± 3 and 66 ± 3 d following first and second postpartum AI were analyzed by logistic

regression using LOGISTIC procedure. Treatment, twin calving, dystocia, retained fetal membranes, metritis and group were offered to all models and retained if $P < 0.2$. The rates at which cows became pregnant up to 200 DIM were analyzed by Cox proportional hazard ratio using the PHREG procedure. Kaplan-Meyer survival analysis using the LIFETEST procedure was used to compare the interval from calving to establishment of pregnancy among treatments.

Statistical significance was defined as $P \leq 0.05$ and statistical tendencies as $0.05 < P \leq 0.10$.

RESULTS

Incidence of Twins, Male Calves, and Postpartum Health Disorders and Milk Yield

The incidences of twins, male calves, and postpartum health disorders of all cows examined for VPD 31 ± 3 d postpartum are depicted in Table 4. Cows diagnosed with VPD at 31 ± 3 d postpartum had greater incidence of dystocia (control = 12.2%, LPS150 = 17.0%, LPS300 = 17.0% and healthy = 3.4%), retained placenta (control = 13.7%, LPS150 = 16.7%, LPS300 = 9.3% and healthy = 1.7%) and metritis (control = 25.5%, LPS150 = 24.1%, LPS300 = 20.4% and healthy = 4.0%) after calving than healthy cows, but there was no difference in incidence of these events among VPD cows only. Incidences of twining (control = 4.1%, LPS150 = 3.8%, LPS300 = 3.8% and healthy = 1.9%) and male calf (control = 44.9%, LPS150 = 49.1%, LPS300 = 43.4% and healthy = 48.0%) were not different between VPD and healthy cows.

There were no (P = 0.97) differences among treatments regarding percentage of first lactation cows enrolled (control = 45.1%, LPS150 = 44.4%, LPS300 = 48.2%, healthy = 44.7%).

Daily milk yield from calving to 200 d postpartum was not (P = 0.22) different among treated and healthy cows (Table 4). Similarly, yield of energy corrected milk (P = 0.27) was not different among treated and healthy cows (Table 4).

Uterine Health and Resolution of Vaginal Purulent Discharge

Counts of PMNL in the uterine cytology slides are presented in Figure 10. Although there was no statistical difference (P = 0.13) among treatments on percentage of PMNL in uterine cytology at baseline, it tended to be greater in control cows (control vs LPS150 P = 0.10; control vs LPS300 P = 0.06; LPS150 vs LPS300 P = 0.83). In addition, there were no differences in percentage of PMNL on days 1, 2 and 7 (P = 0.33), and there was no interaction between treatment and day of sample collection (P = 0.67).

Percentage of cows with > 10% of PMNC in uterine cytology (Table 5) was not different among treatments on d 0 (58.2%; P = 0.23), d 1 (88.3%; P = 0.27), d 2 (84.2%; P = 0.91) or on d 7 (62.6%; P = 0.12). Moreover, percentage of cows with VPD at 7 (P = 0.40; control = 58.0%, LPS150 = 55.8%, LPS300 = 67.9%) and 28 d (P = 0.89; control = 10.6%, LPS150 = 13.7%, LPS300 = 11.8%) after treatment was also not affected by treatment (Table 5).

Reproductive Parameters

Treatment affected pregnancy per AI to first postpartum AI (Table 6). The percentage of cows that were diagnosed pregnant 31± 3 d after the first postpartum AI was lower in for control cows (23.9%) compared to LPS150 (P = 0.03, 42.0%), LPS300 (P = 0.03, 43.8%) and healthy cows (P < 0.01, 49.9%), but pregnancy at 31± 3 d was similar (P > 0.49) among LPS treated cows and healthy cows. Furthermore, likelihood of pregnancy 66 days after first postpartum AI was not different among LPS150 (P = 0.26, 36.0%) or LPS300 (P = 0.17, 35.4%) and healthy cows (46.1%), but control cows were less likely (P < 0.01, 21.7%) to be pregnant at 66 ± 3 days after the first postpartum AI than healthy cows, and tended to have lower pregnancies than LPS150 (P = 0.08) cows. Moreover, there were no differences among treatments in incidence of pregnancy loss from 31 ± 3 to 66 ± 3 d (P = 0.26; control = 8.3%, LPS150 = 17.4%, LPS300 = 18.2%, healthy = 7.2%) after the first postpartum AI, but healthy cows tended (P = 0.07) to have lower pregnancy loss than LPS300 cows. Pregnancy to second postpartum AI was also affected by treatment (Table 6). The percentage of cows diagnosed pregnant 31 ± 3 d after the second postpartum AI was higher for healthy cows (45.0%) than control (P = 0.02, 22.2%) and LPS300 (P = 0.05, 25.0%), but there was no difference in pregnancy 31 ± 3 d after the second postpartum AI between healthy and LPS150 cows (P = 0.47 ; 40.6%) or among VPD cows only (P > 0.19). Similarly, likelihood of pregnancy at 66 ± 3 days after second postpartum AI was higher in healthy cows (40.6%) compared to control (P = 0.05, 22.2%) and LPS 300 (P = 0.02, 16.0%) cows, but pregnancy at 66 ± 3 days was not different between healthy and LPS150 cows (P = 0.67, 38.7%) or among VPD

cows only ($P > 0.11$) (Table 6). Pregnancy loss from 31 ± 3 to 66 ± 3 d after the second postpartum AI was not affected by treatment ($P = 1.00$). Due to small sample size for treated cows (control, n = 8; LPS150, n = 12; LPS300, n = 4), evaluation of pregnancy loss after second postpartum AI was compromised, being 0% for all VPD cows, and therefore the calculation of the adjusted odds ratio was not possible. Pregnancy loss after second postpartum AI was 6.4% (544/1,253) for healthy cows.

Treatment affected ($P < 0.01$) the rate at which cows became pregnant up to 200 d postpartum (Table 6). Pregnancy rate of healthy cows was greater than that of control ($P < 0.01$) and LPS300 ($P = 0.05$) cows, but pregnancy rates of healthy and LPS150 cows were not ($P = 0.16$) different ($P = 0.16$). The median and mean intervals from calving to conception were different among treatments (control = 129 and 123.5 d; LPS150 = 92.5 and 106.1 d; LPS300 = 116 and 111.6 d; healthy = 83 and 100 d; Figure 11).

DISCUSSION

We demonstrated that treatment of cows with VPD at 31 ± 3 DIM with intrauterine infusion of 20 mL of PBS containing 150 μg of *E. coli* LPS improved reproductive performance when compared with cows with VPD that received intrauterine infusion with PBS. Pregnancy at first postpartum AI was not different among LPS150, LPS300, and healthy cows, but control cows were less likely to be pregnant after first postpartum AI than healthy cows. Moreover, pregnancy at second postpartum AI and pregnancy rate up to 200 DIM was not different between LPS150 and healthy cows. The improvement in pregnancy to first postpartum AI resulting from the LPS intra-uterine is

very significant particularly because cows diagnosed with VPD were also more likely to have peripartum diseases such as dystocia, retained fetal membranes, and metritis that are known to reduce pregnancy per AI. Retained placenta and metritis for instance, are reported to reduce conception rate and to extend intervals to pregnancy (Goshen and Shpiegel, 2006), and dystocia has been reported to increase number of services and days open (Dematawewa and Berger, 1997).

The improvement in reproductive performance of cows with VPD treated with intra-uterine infusion with LPS observed in the present study is in accordance with findings reported by Singh et al. (2000). It is worth noting that Singh et al. (2000) treated endometritic cows on the day they were observed in estrus expression, whereas in the current experiment cows were treated independently of phase of estrus cycle. Treating endometritic cows on the day of estrous expression could potentiate LPS immunomodulatory action because cows have lower progesterone concentrations during estrus, reducing the immunosuppressant effects of progesterone (Siiteri et al., 1977; Hansen, 2013). On the other hand, treatment of endometritic cows expressing estrus or on the day of AI would be difficult to implement in a commercial dairy farm. Secondly, the treatment would be limited to the cows that exhibit estrus signs, and estrus expression in endometritic cows is compromised as postpartum uterine infection negatively affects ovarian function (Sheldon et al., 2002; Williams et al., 2007; Williams et al., 2008; Sheldon et al., 2009).

Lipopolysaccharide did not cause systemic sickness in treated cows. Williams et al. (2008) infused 3 μg/kg of LPS in the uterus of Holstein heifers and observed no

detrimental effect on rectal temperature or concentration of alpha-1acid glycoprotein. However, fewer heifers treated with LPS ovulated compared with heifers treated with PBS and, among heifers that ovulated, progesterone concentration was lower in heifers treated with LPS compared with those treated with PBS.

Despite improvements in reproductive performance observed in LPS treated cows, no differences among treatments in resolution of VPD 7 and 28 d after treatment were observed. Moreover, there were no differences among treatments in incidence of subclinical endometritis 7 d after treatment. Therefore, we speculated that there was an improvement in uterine health posterior to the first week after treatment, but the metricheck technique performed on d 28 may not have been sensitive enough to detect improvements in uterine condition in LPS treated cows. The number of PMNL in the uterine cytology increased in approximately 60% in LPS treated cows within 24 h after treatment and then returned to pre-treatment counts by 7 d after treatment. On the other hand, number of PMNL in uterine cytology of control cows remained relatively constant from pre-treatment to 7 d after treatment. More experiments are necessary to determine if the fast influx of PMNL into the uterus resulting from LPS infusion resolves mild uterine infections and improves uterine health beyond 7 d after treatment. Even though the mechanism by which LPS improved fertility is not completely clear, LPS treatment increased percentage of PMNL positive for phagocytosis and oxidative burst, and increased intensity of expression of CD18 by PMNL (Moraes et al., 2013). The CD18 adhesion molecule is a member of the β2-integrins subfamily and plays important role in the PMNL firm adhesion to and diapedesis through endothelium during inflammation

(Carlos and Harlan, 1994). Thus, increased CD18 expression in PMNL of LPS treated cows may explain the greater number of PMNL in uterine cytology of LPS treated cows 24 h after intra-uterine infusion. This increased PMNL expression of CD18, phagocytosis and oxidative burst, may be caused by LPS intra-uterine infusion and production of pro-inflammatory cytokines and chemokine (TNFα, IL-1, IL-6, IL-8; Wright, 1999). Endometrial mRNA expressions of adhesion molecules (E-selectin, ICAM-1 and VCAM-1), inflammatory cytokines (IL-1β, IL-6, IL-8 and IL-10) and TLR-4 at 6 and 24 h after treatment were not different among LPS150, LPS300, and control cows (Moraes et al., 2013a). It is possible that the timing of endometrial biopsy was not ideal to demonstrate effects of LPS treatment on regulation of mRNA expression of such molecules, since peak mammary mRNA expression of TNF-α after LPS intra-mammary infusion occurred at 3 h after treatment and values were back to baseline values by 12 h after treatment (Schmitz et al., 2004).

CONCLUSIONS

Treatment of VPD cows with intrauterine infusion of 150 μg of *E. coli* LPS improved reproductive performance compared with VPD cows that treated with PBS or 300 μg of *E. coli* LPS intrauterine infusion. It is possible that the high influx of PMNL resulting from LPS intrauterine treatment resulted in clearing of intrauterine infections and improvement of uterine health. The lack of differences in cure from VPD may be because of the poor accuracy of Metricheck to determine intrauterine health. More

experiments are needed to elucidate more clearly the mechanisms through which intrauterine LPS treatment improves pregnancy per AI of cows with VPD.

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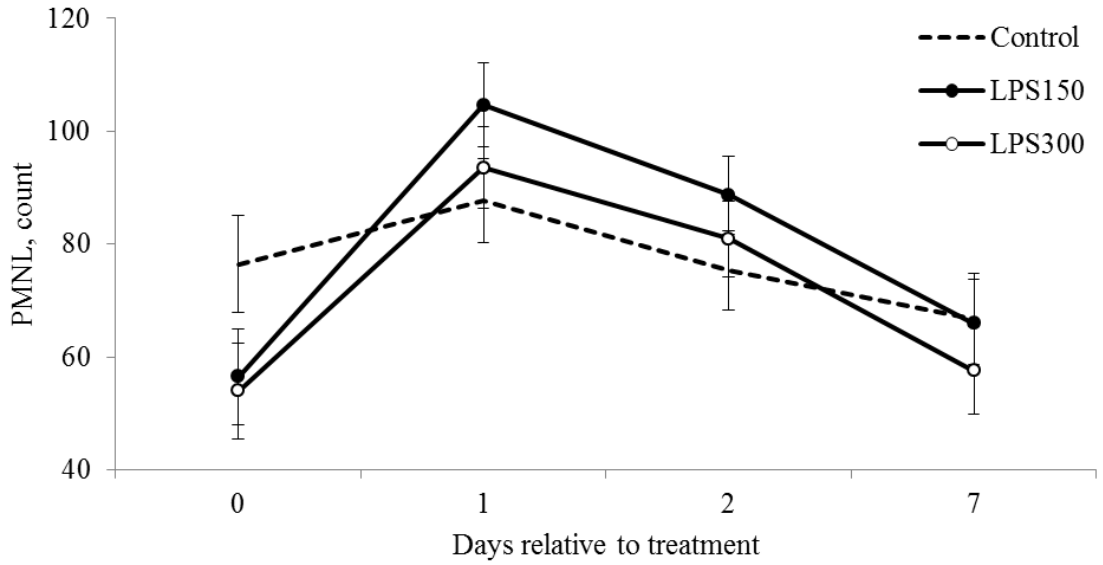

Figure 10. Effect of LPS intrauterine treatment on count of PMNL from endometrial cytology. Treatments: Control - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of *E. coli* LPS as treatment; LPS300 - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of *E. coli* LPS as treatment. ** PMNL count tended to be greater in control cows than LPS150 (P = 0.10) and LPS300 (P = 0.06) cows. There were no differences in counts of PMNL on days 1, 2 and 7 (P = 0.33), and there was no interaction between treatment and day of sample collection (P = 0.67).

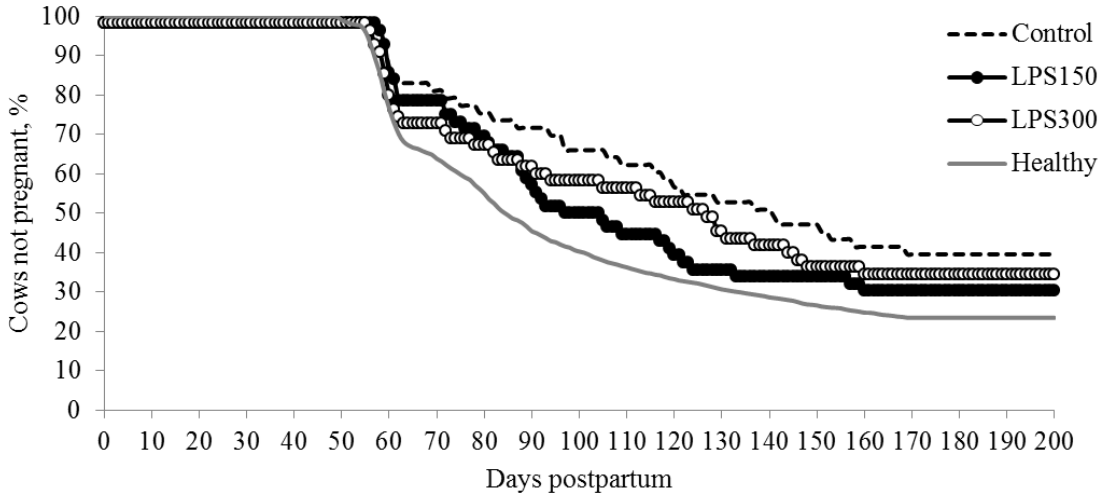

Figure 11. Effect of LPS intrauterine treatment on survival distribution of time to pregnancy up to 200 days postpartum. Effect of treatment according to the Wilcoxon test of equality: P < 0.01. Treatments: Control - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of *E. coli* LPS as treatment; LPS300 - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of *E. coli* LPS as treatment; Healthy - composed by healthy cows at Metricheck examination (less than 50% of pus in retrieved exudate). Pregnancy rate of healthy cows was greater than that of control [$P < 0.01$; AHR (95% CI) = 0.59 (0.41, 0.84)] and LPS300 [$P = 0.05$; AHR (95% CI) = 0.71 (0.50, 0.99)] cows, but pregnancy rate of healthy and LPS150 cows was not different [$P = 0.16$; AHR (95 % CI) = 0.79 (0.57, 1.10)].

Table 4. Incidence of postpartum problems among cows diagnosed with vaginal purulent discharge and health cows.

Items	Treatments				*P* - value
	Control[2]	LPS150[2]	LPS300[2]	Healthy[2]	
Percentage of first lactation cows, %	45.1	44.4	48.2	44.7	0.97
Twins, AOR (95 % CI)[1]	2.24 (0.53, 9.49)	2.01 (0.49, 8.73)	2.01 (0.49, 8.73)	Ref.	0.40
Male calf, AOR (95 % CI)[1]	0.88 (0.50, 1.56)	1.04 (0.61, 1.80)	0.83 (0.48, 1.44)	Ref.	0.89
Dystocia, AOR (95 % CI)[1]	3.94 (1.63, 9.49)	5.77 (2.73, 12.19)	5.77 (2.73, 12.19)	Ref.	< 0.01
Retained fetal membranes, AOR (95 % CI)[1]	9.26 (3.96, 21.67)	11.64 (5.37, 25.24)	5.939 (2.26, 15.61)	Ref.	< 0.01

Metritis, AOR (95 % CI)[1]	8.17 (4.23, 15.80)	7.58 (3.94, 14.56)	6.11 (3.07, 12.19)	Ref.	< 0.01
Milk yield, kg/d	30.02 ± 1.09	32.05 ± 1.05	31.34 ± 1.07	30.12 ± 0.16	0.22
Yield of energy corrected milk, kg/d	34.34 ± 1.14	36.60 ± 1.10	35.83 ± 1.11	34.70 ± 0.17	0.27

[1] AOR = Adjusted odds ratio; 95% CI = 95% confidence interval

[2] Treatments: **C**ontrol - **c**ows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 μg of *E. coli* LPS as treatment; LPS300 - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 μg of *E. coli* LPS as treatment; Healthy - composed by healthy cows at Metricheck examination (less than 50% of pus in retrieved exudate).

Table 5. Intrauterine cell population and resolution of vaginal purulent discharge following treatment.

	Treatment			*P* – value
	Control[1]	LPS150[1]	LPS300[1]	
Cows with > 10% of PMNL in uterine cytology, % (n)				
d 0 before treatment	66.7 (51)	58.5 (53)	50.0 (54)	0.23
d 1 after treatment	84.0 (50)	94.2 (52)	86.5 (52)	0.27
d 2 after treatment	82.4 (51)	84.9 (53)	85.2 (54)	0.91
d 7 after treatment	72.3 (47)	64.0 (50)	52.0 (50)	0.12
Cows with VPD 7 d after treatment, AOR (95 % CI)[1]	Ref.	0.91 (0.42, 2.00)	1.53 (0.69, 3.43)	0.40
Cows with VPD 28 d after treatment, AOR (95 % CI)[1]	Ref.	1.34 (0.39, 4.54)	1.12 (0.32, 3.95)	0.89

[1] AOR = Adjusted odds ratio; 95% CI = 95% confidence interval

[2] Treatments: Control - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of *E. coli* LPS as treatment; LPS300 - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of *E. coli* LPS as treatment.

Table 6. Effects of LPS treatment on percentage of cows pregnant after first and second postpartum AI, pregnancy loss for first and second AI and rate at which cows became pregnant up to 200 d postpartum.

	Treatment				P – value
	Control[3]	LPS150[3]	LPS300[3]	Healthy[3]	
First postpartum AI, AOR (95 % CI)[1]					
P/AI 31 ± 3 d after AI	0.30 (0.15, 0.61)[a]	0.81 (0.45, 1.46)[b]	0.81 (0.45, 1.46)[b]	Ref.[b]	< 0.01
P/AI 66 ± 3 d after AI	0.30 (0.14, 0.64)[a,A]	0.71 (0.39, 1.29)[B]	0.66 (0.36, 1.20)	Ref.[b]	< 0.01
Pregnancy loss from 31 ± 3 to 66 ± 3 d after AI	1.09 (0.13, 9.38)	1.97 (0.54, 7.22)	2.86 (0.91, 8.99) [A]	Ref.[B]	0.26
Second postpartum AI, AOR (95 % CI)[1]					
P/AI 31 ± 3 d after AI	0.37 (0.17, 0.83)[a]	0.76 (0.36, 1.60)	0.41 (0.17, 0.99)[a]	Ref.[b]	0.02
P/AI 66 ± 3 d after AI	0.45 (0.20, 1.00)[a]	0.85 (0.40, 1.82)	0.29 (0.10, 0.85)[a]	Ref.[b]	0.03

Pregnancy rate up to 200 d, AHR(95% CI)[2]	0.59 (0.41, 0.84)[a]	0.79 (0.57, 1.10)	0.71 (0.50, 0.99)[a]	Ref.[b]	< 0.01

[a,b] Within a row, numbers with lowercase letters (a,b) are different ($P < 0.05$)

[A,B] Within a row, numbers with uppercase letters (A,B) tended ($0.05 < P \leq 0.10$) to be different.

[1] AOR = Adjusted odds ratio; 95% CI = 95% confidence interval

[2] AHR = Adjusted hazard ratio; 95% CI = 95% confidence interval

[3] Treatments: Control - cows with vaginal purulent discharge (VPD) that received intrauterine infusions of 20 mL of PBS as treatment; LPS150 - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 150 µg of *E. coli* LPS as treatment; LPS300 - cows with VPD that received intrauterine infusions of 20 mL of PBS containing 300 µg of *E. coli* LPS as treatment; Healthy - composed by healthy cows at Metricheck examination (less than 50% of pus in retrieved exudate).

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